DEVELOPMENTAL REGULATION OF CONSTITUTIVE AND ANGIOTENSIN II-INDUCED PROTEIN SECRETION BY ASTROCYTES OF THE BRAIN

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1990

To the memory of Francis Joseph Skupin

ACKNOWLEDGEMENTS

This dissertation is the result of diligence and commitment of several truly outstanding individuals. Sincere thanks go to my mentors, Drs. Kathleen T. Shiverick and Mohan K. Raizada, for their guidance, both professional and personal. I am truly fortunate to have had the input of these two excellent scientists in my education and scientific development. I similarly wish to thank the members of my graduate committee, Dr. Stephen Baker, Dr. William Dunn and especially Dr. William Buhi who graciously offered both his laboratory resources and biochemical expertise to me (I believe there to be no protein which can escape his focus, except on Thursday nights).

Heartfelt gratitude goes to my friends and colleagues Dr. Laura Mudd, Elizabeth Albert, Terry Medrano, Dr. David Kroll, Dr. Paul Kroeger, Jeff Lawrence, Chris Borgert, Dr Michael Moser and Idonia Alvarez for their advice and assistance. I am particularly indebted to Dr. Susan Ogilvie and to Stacie Cohen for their friendship, help (to put it mildly) and encouragement during these last two years. Special thanks go to my colleagues in the M.D.-Ph.D. program, especially Mike Huie and Dr. Harry Ostrer who have reminded me countless times of the importance of art and philosophy in science. I also wish to thank the secretarial staff of the Department of Pharmacology and Therapeutics, especially Judy Adams, for their assistance.

Finally, I wish to acknowledge three people I admire most. I am thankful for my loving parents, John and Patricia, who have always placed the concerns of my brothers and me before their own. Beyond all others, I will ultimately owe each of my triumphs in life to my Mom and Dad. I also wish to thank Ms. Kimberly Ann Hillner, my future wife, for her love, understanding and boundless patience for the idio-syncrasies of this scientist. With her, I look forward to a lifetime of discovery.

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December, 1990

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Astrocytes of the brain are believed to play an important role in growth, development and maintenance of differentiated function of cells in the mammalian central nervous system. We hypothesize that astrocytes cultured from brain demonstrate developmental changes in secretion of proteins which may mediate interactive processes between these and other cells of the developing brain. We further believe that CNS sensitivity to certain centrally active peptides during development may be manifested by induction of protein secretion by astrocytes. This study investigated both changes in secretion of proteins by cultured CNS astrocytes of postnatal developing rat brain, and changes in sensitivity of these cells to Angiotensin II, a centrally-active peptide with unknown actions on astrocytes. Results

of this study indicate that astrocytes cultured from maturing rat brain secrete significantly higher levels of insulin-like growth factor (IGF) binding protein, IGFBP-2, than those cultured from neonatal rat brain. Age-dependent differences in secretion of IGFBP-2 by astrocytes correlate well with IGFBP-2 messenger RNA levels detected in these cells. These studies also show that the differentiating agent, diButyryl 3', 5'-cyclic adenosine monophosphate induces secretion of IGFBP-2 from 21-day rat brain astrocytes, an event associated with striking changes in morphology of these cells. Studies presented here also indicate that angiotensin II (Ang II) binds to specific cell surface receptors on 21-day rat brain astrocytes to stimulate DNA and protein synthesis, and induce synthesis and secretion of two proteins, plasminogen activator inhibitor-1 and a tissue inhibitor of metallo-proteases-related protein. These studies extend the role of astrocytes in developing brain to include modulation of IGF activity via secretion of specific IGF binding proteins and identify Ang II as both a growth factor for CNS astrocytes and an inducer of protease inhibitory activity in these cells.

CHAPTER 1
INTRODUCTION

> Men ought to know that from the brain only, arise our pleasures, joys, laughter and jests, as well as our sorrows, pains griefs and tears. Through it, in particular, we think, see, hear, and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant....
> *-Hippocrates, Fifth Century, B.C.*

The brain, simultaneously responsible for the detection and integration of environmental stimuli, coordination of multiple physiologic processes, and generation of cognitive states, must possess an almost incomprehensible level of sophistication. Indeed, the mammalian central nervous system (CNS) is the most complex of all tissues with estimates of its cellular content reaching 10^9 and higher. In addition to their immense numbers, many types and subtypes of cells are present in the CNS each distinct with respect to both developmental programme' and differentiated function.

Despite its inherent complexity, development and differerentiation of the cellular constituents of the nervous system seem to follow commonly-held principles which are now coming into focus. Primary embryonic induction is the first in a series of critical events in the development of the nervous system. During gastrulation, invading mesoderm signals the overlying ectoderm to commit to neural

development, and cells in "committed fields" of the resulting neuroectoderm form different parts of the nervous system. Developmental decisions taken by these neuroepithelial precursors are thought to define the type, number and spatial organization of cells in the adult brain (McKay, 1989). Thus, a central issue in developmental neurobiology is to identify specific progenitor cells in an apparently homogeneous population of neuroepithelial cells and understand mechanisms by which they give rise to the great diversity of cells in the mature CNS. This issue is especially pertinent to the study of the most populous cells of the brain, astroglia.

Astrocytes and Cellular Growth and Differentiation in the CNS

A useful model for the study of glial cell diversification has been the rat optic nerve (Raff, 1989). This nerve is amenable to relatively controlled study of glial populations since no endogenous neuronal cell bodies are present. *In vitro* studies of developing optic nerve have identified two distinct glial cell lineages which give rise to three antigenically and functionally separate cell types in the adult optic nerve. Oligodendrocytes, cells responsible for myelination of neuronal axons and type-2 astrocytes, whose processes extend to nodes of Ranvier, develop from a common, bipotential O-2A progenitor cell (Raff *et al.*, 1983). Type-1 astrocytes, in contrast, are thought

to develop from a distinct precursor cell (Raff *et al.*, 1984). In addition to demonstrating distinct glial cell lineages in the CNS, these experiments have shown that the appearance of these three glial cell types *in vivo* follows distinct temporal patterns. Studies in which antibodies were used to distinguish the three glial cell types in cell suspensions prepared from optic nerves of developing rats demonstrate that type-1 astrocytes first appear at embryonic day 16 (E16), oligodendrocytes on the day of birth (usually E21), and type-2 astrocytes at 10-14 days after birth (Miller *et al.*, 1985). These studies and others indicate that the ontogeny of glial cells of the CNS may follow distinct temporal and spacial patterns not unlike those of the hematopoeitic system and, in fact, the term "neuropoeisis" has been applied to this process by several investigators (Anderson, 1989). Moreover, analyses of glial cell diversification in the brain may provide clues to origins of other CNS cell types, especially neurons. Both retroviral and fluorescent dextran lineage marking experiments indicate that CNS astrocytes and neurons may share common precursors in the retina and elsewhere in the CNS (Turner and Cepko, 1987; Wetts and Fraser, 1988).

An unresolved issue in discussions of CNS cellular differentiation and development is the role of inherent cellular programming versus environmental stimuli in determining developmental decisions made by CNS progenitor cells. In the former possibility, ancestry is of primary

importance to precursor cells which generate products through a fixed lineage of cell division and differentiation. In the latter, cellular microenvironment dictates the pattern of differentiation of multipotential stem cells. This type of model, analogous to that of the hematopoeitic system, places great emphasis is placed on cell-cell interactions. Here, astrocytes may play a central role.

CNS astrocytes, long considered merely passive support elements for neurons, have only recently been recognized as active participants in the development and maintenance of normal brain physiology. During CNS development, radial glial cells serve not only as physical guides to neuronal cell migration from the periventricular proliferative zone, but may also induce neuronal differentiation and actually form the structural plan for the brain (Rakic, 1972; Rakic, 1988). Astrocytes also promote growth and maintenance of differentiated neurons *in vitro* (Banker, 1989; Muller and Seifert, 1982; Patel and Hunt, 1989; Rudge *et al.*, 1985). Type-1 astrocytes promote division and inhibit premature differentiation of O-2A progenitor cells (Noble *et al.*, 1988; Raff *et al.*, 1985) as well as induce blood-brain barrier properties in cerebral capillary endothelial cells (Risau, 1986; Risau *et al.*, 1986b; Janzer and Raff, 1987).

Although well-established anatomical relationships between astrocytes, neurons and endothelial cells suggest interactive processes between these cells, little is currently understood of glial-neuronal, glial-endothelial or

glial-glial communication in the developing and mature brain, or of factors which may mediate such interaction. However, an increasing body of evidence is beginning to implicate astrocyte-derived peptides as critical components of intercellular communication within the CNS.

Astrocyte-derived Peptides as Mediators of Intercellular Communication in the Brain

Until recently, interest in peptide factors which promote growth and differentiation of CNS cellular elements has focussed primarily on peptides capable of promoting neuronal survival, the so-called neurotrophic factors. Suprisingly, the first and best-characterized peptide factor important for neuronal growth and development in the CNS was discovered not in astrocytes or in brain, but in the male mouse submaxillary gland. First and perhaps best evidence for a role of nerve growth factor (NGF) in neuronal survival came from experiments in which selective neuronal death occurred in the peripheral sympathetic nervous system of newborn rodents injected with anti-NGF antibodies (Cohen, 1960). Subsequent investigation further indicated that NGF was secreted in limited quantities by targets of innervation and decreased the well-known phenomenon of naturally occuring cell death of neurons in both sympathetic and dorsal root ganglia (DRG) (Korsching and Thoenen, 1983; Oppenheim *et al.*, 1982; Hamburger *et al.*, 1981). For over twenty years, these and other studies of NGF action provided substantial evidence

that trophic peptides are important to neuronal growth and survival. Yet, only within the last five years have observations of NGF activity in peripheral systems been extended to include the CNS. In the brain, NGF has now been shown to promote the survival of adult cholinergic neurons projecting from the basal forebrain nuclei to the hippocampus and cortex (Hefti, 1987).

Despite NGF's classical description as a target neuron-derived trophic factor, several lines of evidence indicate a glial source of this peptide as well (Lindsay, 1979; Schwartz *et al.*, 1977; Westermann *et al.*, 1988). Furthermore, several other target-derived neurotrophic factors have been identified which may also be secreted by astrocytes. These include brain-derived neurotrophic factor (Barde *et al.*, 1982), insulin-like growth factors (Recio-Pinto and Ishii, 1984a), basic fibroblast growth factor (Unsicker *et al.*, 1987; Hatten *et al.*, 1988; Petteman *et al.*, 1985) and glial-derived protease nexin-1 (Gloor *et al.*, 1986; Gurwitz and Cunningham, 1988).

Brain-derived neurotrophic factor (BDNF) is a 12,500 M_r basic protein, closely related to NGF in terms of biochemical properties, amino acid sequence, and receptor binding characteristics (Barde, 1989). Like NGF, BDNF prevents naturally occurring neuronal death in the dorsal root ganglion (DRG) at early stages *in vivo* (Hofer and Barde, 1988), yet once this period of naturally occurring cell death is over (E9), BDNF and NGF become complementary with respect

to their target neuronal populations in the DRG. BDNF also induces neurite outgrowth in isolated adult rat DRG neurons suggesting that BDNF responsiveness is not confined to prenatal development (Lindsay, 1988). At present, the cellular source of BDNF is unknown; however, its similarity to NGF make astrocytes possible candidates.

Insulin and the insulin-like growth factors, IGF-1 and 2 are structurally related to NGF and demonstrate distinct neurotrophic properties (Ishii *et al.*, 1989). Physiologically relevent concentrations of IGF-2 increase both the proportion of cells with neurites and the length of such neurites in neuroblastoma cells of sympathetic origin (Recio-Pinto and Ishii, 1984a) as well as sensory and sympathetic neurons in culture (Recio-Pinto *et al.*, 1986). Subnanomolar concentrations of IGF-1 enhance survival of fetal CNS neurons in culture (Aizenman and de Vellis, 1987) as well as support neurite outgrowth in embryonic spinal cord cells (Ishii *et al.*, 1989). IGF-1, with NGF, may also promote survival of cholinergic neurons in postnatal animals. IGF-1 specifically increases potassium-dependent release of [^{3}H]-acetylcholine release from adult rat cortical slices (Nilsson *et al.*, 1988) and stimulates choline acetyltransferase activity in mixed cultures of neonatal neurons.

The acidic and basic fibroblast growth factors (aFGF and bFGF) are relatively abundant 16,000 M_r peptides present in both the fetal and adult CNS (Risau *et al.*, 1988;

Godspardowicz, 1987). In addition to their mitogenic role in several nonneuronal cell types, these growth factors promote *in vitro* survival of a wide variety of embryonic neurons (Barde, 1989) and their infusion into ventricles can rescue adult cholinergic neurons *in vivo* following fimbria-fornix lesion (Anderson *et al.*, 1988). FGF appears to be released by astrocytes *in vitro* since addition of bFGF to cultured cerebellar granule cells mimics effects of co-cultured astrocytes on survival and neurite outgrowth from these cells, while anti-bFGF antibodies prevent neurite extension from these cells in the presence of astrocytes (Hatten *et al.*, 1988).

Glial-derived protease nexin I (GdPN-1) is a M_r 43,000 serine protease inhibitor (serpin) isolated from conditioned medium of C-6 rat glioma cells (Geunther *et al.*, 1985). This protease inhibitor induces morphologic differentiation of neuroblastoma cells (Monard *et al.*, 1973) and stimulates proliferation of mouse cerebellar astrocytes and astrocyte precursors in a dose-dependent manner (Gloor *et al.*, 1986). A protein with the same characteristics as GdPN-1 is detected following injury to nerves of the regenerating peripheral nervous system but is not found following similar injury to the nonregenerating CNS, suggesting that this peptide may be linked to axonal regeneration *in vivo* (Patterson, 1985). Observations that GdPN-1 is a potent serine protease inhibitor complexing with proteases such as urokinase, tissue plasminogen activator, thrombin and trypsin, (Geunther *et*

al., 1985) and that other serine protease inhibitors (hirudin and the synthetic tripeptide D-Phe-Pro-ArgCH_2Cl) promote neurite outgrowth suggest that neurotrophic properties of this peptide relate to its protease inhibitory activity (Monard *et al.*, 1983). That neither hirudin nor D-Phe-Pro-ArgCH_2Cl cause proliferation of astrocytes suggest that protease inhibitory activity of GdPN-1 alone is insufficient for its mitogenic properties (Gloor *et al.*, 1986). Unlike many characterized neurotrophic factors which are found in a variety of tissues and act predominantly during embryonic and fetal development, GdPN-1 is restricted to brain tissue and appears to be developmentally regulated in the postnatal rat (Gloor *et al.*, 1986).

Excluding NGF, FGFs and GdPN-1, few other astrocyte-derived trophic factors for CNS neurons have been identified unambiguously in astrocytes; however, several observations suggest their existence. Neuronal survival *in vitro* is greatly enhanced when these cells are grown in the presence of astrocytes (Touzet and Sensenbrenner, 1978) or astrocyte-conditioned culture medium (Muller *et al.*, 1982; Banker, 1980). The neurotrophic properties of two peptides, vasoactive intestinal peptide (VIP) and epidermal growth factor (EGF), are indirect and seem to be mediated by release of unidentified neurotrophic substances by astrocytes (Wang *et al.*, 1989; Morrison *et al.*, 1987; Brenneman *et al.*, 1987). C6 glioma cells secrete up to seven distinct neurotrophic factor-like "activities" as demonstrated by *in vitro* bioassay

of conditioned culture medium from these cells (Westermann *et al.*, 1988). These studies clearly suggest that astrocyte and astrocyte-derived cell lines synthesize and secrete several factors *in vitro* that are involved in growth and maintenance of CNS neurons.

Although much work in the area of CNS regulatory peptides has been devoted to identification and characterization of neurotrophic factors, substantial evidence points to the existence of peptide growth and differentiation factors whose target cells may include other cells of the brain including cerebral capillary endothelial cells, ependymal cells, oligodendrocytes and astrocytes.

Extracts from embryonic chicken brain contain an angiogenic factor which has been determined by gel-filtration to be a protein of 16-18 kDa (Risau, 1986a). Partially purified preparations of this factor stimulate proliferation of chorio-allantoic endothelial cells as well as induce expression of a 74 kDa protein specific to endothelium forming the blood-brain barrier (Risau *et al.*, 1986b). Although the cellular origin of this peptide remains obscure, astrocytes seem to be the most likely candidates since their processes form endfeet which surround the CNS microvasculature. That astrocytes secrete such "angiotrophic" factors is a plausible hypothesis since purified populations of type-1 astrocytes promote proliferation of, and induce blood-brain barrier properties

in, chorio-allantoic endothelial cells *in vivo* (Janzer and Raff, 1987).

Insulin and the IGFs are promoters of oligodendrocyte development and may therefore play a role in myelination in the CNS. Evidence for such a role of insulin and IGF-1 includes observations that exposure of mixed brain cell cultures to IGF I stimulates 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), an oligodendrocyte-specific enzyme (Lenoir and Honeggar, 1983). IGF-1 treatment of mixed brain cell cultures grown in the presence of 10% fetal bovine serum increases the number of cells expressing both CNPase and galactocerebroside (GC), another oligodendrocyte-specific antigen (McMorris *et al.*, 1986; Raff *et al.*, 1978). Insulin also induces CNPase in these cultures (McMorris, 1983). IGF I also induces myelin basic protein synthesis in purified populations of oligodendrocytes in culture (Saneto *et al.*,1988). While IGF I clearly induces several parameters of oligodendrocyte differentiated function, physiological sources of this peptide in adult brain may be uncertain (Latteman *et al.*, 1989). Astrocytes may secrete other factors which regulate oligodendrocyte activity. Conditioned medium from purified populations of type-1 astrocytes stimulate proliferation of O-2A oligodendrocyte progenitor cells in cultures of optic nerve from both embryonic and perinatal rats (Noble *et al.*, 1988; Raff *et al.*, 1988). Thus, it is thought that oligodendrocyte differentiation depends upon an intrinsic clock in the O-2A progenitor cell

which is driven by growth factor (or factors) secreted by type-1 astrocytes. Observations that platelet-derived growth factor (PDGF) can mimic this activity and that anti-PDGF antibodies can inhibit the effect of type-1 astrocyte-conditioned medium in these experiments indicate that glial-derived PDGF is at least partly responsible for driving the O-2A progenitor to the oligodendrocyte lineage (Noble *et al.*, 1988; Raff *et al.*, 1988). Type-1 astrocytes also inhibit *in vitro* myelination of dorsal root ganglion axons by oligodendrocytes purified from adult rats (Rosen *et al.*, 1989). This effect is mimicked by type-1 astrocyte-conditioned medium again suggesting that glial-derived soluble factors play an important role in myelination in the CNS. Interestingly, observations from our laboratory and others (Snipes *et al.*, 1987) suggest that astrocytes *in vitro* produce large amounts of apolipoprotein E, a protein capable of binding free lipids. The astrocyte inhibitory effect on myelination may then be due, at least in part, to production of this protein which could limit axonal myelination by oligodendrocytes at the level of substrate availability (Ignatius *et al.*, 1987).

Studies in recent years have also established the presence of peptide factors in brain which are involved in both the stimulation and inhibition of astroglial cell growth. These "brain-derived factors" are often identical to known peptides in other tissues and include, EGF, the interleukins (IL-1 and 2), and IGF-1 and 2.

EGF is a small polypeptide purified from the same source as NGF, and has been most extensively studied as both a mitogen and promoter of differentiation in cells of mesodermal and ectodermal origin (Carpenter and Cohen, 1979). In recent years, EGF has become one of the best characterized mitogenic peptides in the CNS. Receptors for EGF (EGF-R) are abundant on cultured astrocytes, few in number on oligodendrocytes and absent in cultured neurons.(Simpson *et al.*, 1982; Leutz and Schachner, 1982; Wang *et al.*, 1989). Occupation of astrocyte receptors by EGF causes marked proliferative responses in cultures these cells including DNA, RNA and protein synthesis (Avola *et al.*, 1988a; Avola *et al.*, 1988b), as well as increases in astrocyte cell number (Wang *et al.*, 1989). EGF also induces astrocyte expression of several growth-associated genes, including the proto-oncogene c-fos (Condorelli *et al.*, 1989; Arenander *et al.*, 1989). Several observations also support a role for EGF in astrocyte growth regulation *in vivo*. In adult rats, EGF receptor immunoreactivity in astrocytes is low but increases dramatically in areas of reactive gliosis following CNS lesion. Furthermore, a soluble astrocyte mitogen inhibitor, immunologically related to the EGF receptor and thought to inhibit EGF mitogenic action under normal physiological conditions, is reduced by up to 50% in areas of reactive gliosis following CNS injury (Nieto-Sampedro, 1988). Evidence also suggests that the EGF/EGF-R system is functionally disturbed in CNS tumors of astrocytic origin.

Amplification of an altered EGF receptor gene (c-erbB) results in a ligand-independent increase in EGF receptor tyrosine kinase activity in glioblastome multiforme (Yamazaki *et al.*, 1988). Overexpression of EGF receptors is often found in other types of glioma (Liberman *et al.*, 1984; Liberman *et al.*, 1985).

The presence of immunomodulatory peptides (IL-1 and 2) which regulate astrocyte proliferation was demonstrated following detailed accounts of cellular responses to CNS trauma. Following brain injury, both astrocytes and microglia, phagocytic cells derived from the monocyte/macrophage lineage, undergo marked proliferation and morphological change resulting in formation of the well-known glial scar (Kitamura, 1980; Latov *et al.*,1979). This trauma-induced "reactive gliosis" can be inhibited by immunosuppression with methotrexate (Billingsley, *et al.*, 1982). These observations along with those indicating that mitogen-stimulated T-lymphocytes produce a glial stimulatory factor which induces proliferation, DNA synthesis, and RNA synthesis in cultures of fetal rat brain astrocytes are suggestive of a role of lymphokines in reactive gliosis. Indeed, IL-1 and 2, peptides which regulate cell division and maturation in the immune system, are present in high levels following brain trauma (Nieto-Sampedro and Berman, 1987a; Nieto-Sampedro and Chandy, 1987b). IL-1 promotes astrocyte cell division, [^{3}H]-thymidine incorporation and accelerated expression of glial fibrillary acidic protein (GFAP) in

embryonic astrocyte progentior cells (Giulian *et al.*, 1988a). Although cellular origin(s) of IL-1 *in vivo* is uncertain, this peptide is secreted *in vitro* by both ameboid microglia (Giulian *et al.*, 1986) and astrocytes (Nieto-Sampedro and Berman, 1987a). The main target of IL-2 in the CNS seems to be oligodendrocytes (Benveniste and Merrill, 1986) but the cellular source of this peptide in brain is unknown.

IGF-1 induces proliferative responses in neonatal rat brain astrocyte similar to those demonstrated in peripheral tissues. Subnanomolar concentrations of IGF-1 stimulate incorporation of [^{3}H]-thymidine in serum-deprived cultures of astrocytes (Shemer *et al.*, 1987) and insulin produces a similar effect, but at 100-fold higher concentrations, suggesting that this response is probably mediated by occupation of IGF-1 receptors.

Astrocytes also secrete peptide factors involved in autocrine mechanisms of growth regulation but, as in the case of neurotrophic factors, few of these factors have been identified precisely. Serum-free conditioned medium from rat cerebellar astrocytes inhibits [^{3}H]-thymidine incorporation into proliferating astrocytes, an effect attributed to an unknown peptide present in a molecular weight fraction, M_r > 10,000, of this medium (Aloisi *et al.*, 1987). Similar studies have identified other glial regulatory factors including two astroglial growth factors similar to fibroblast growth factors (Petteman *et al.*, 1985), glia maturation factor ß (Lim *et al.*, 1989) and glial growth factor (Lemke

and Brockes, 1984). Cellular sources of these peptides are at present unknown but, based upon previous examples, astrocytes may be a likely source.

Current evidence thus suggests that growth, development and maintenance of differentiated function of the various cell populations of the CNS are tightly regulated by growth factors produced, at least in part, by brain astrocytes. As exemplified by the interleukins 1 and 2, an increasing number of peptide hormones have joined the ranks of traditional growth factors in regulation of cellular growth and development in the CNS. Investigations into the identity, cellular origin and mechanism of action of these factors will surely contribute to a greater understanding cellular interactions in the CNS.

Angiotensin II: A Peripheral Hormone Finds New Roles in the Brain

General Considerations of the Renin-Angiotensin System

The octapeptide angiotensin II (Ang II) is the principle bioactive product of the renin-angiotensin system (RAS) implicitly involved in regulation of blood pressure, fluid and electrolye homeostasis (Catt *et al.*, 1984). The biosynthetic pathway for angiotensin peptides has been well characterized. Angiotensinogen, the 45,000 M_r parent molecule of Ang I and II, is synthesized by liver and circulates unbound in plasma. Cleavage of this peptide by

renin secreted from the juxtaglomerular apparatus of the kidney yields the decapeptide, Ang I. This prohormone is rapidly converted to the octapeptide, Ang II, by a ubiquitous converting enzyme present in high amounts in lung. Ang II subsequently binds to receptors on target cells to exert its physiological effects.

Considerable evidence now suggests that this classical model of the RAS must be modified to delineate the effects of multiple tissue RAS systems from those of the circulating RAS (reviewed in Campbell, 1987). Such local RAS systems have been demonstrated in brain, kidney, adrenal, testis and arteriolar wall where they have been proposed to work in concert with the circulating RAS. Contrary to the classical model, this model emphasizes that the primary role of the systemic RAS is the delivery of angiotensoinogen and renin to tissues, not Ang II. These systemic substrates then act locally with tissue converting enzyme to generate Ang II which interacts with target cells via specific Ang II receptors. This is an attractive model since it allows for local tissue-specific modulation of RAS activity.

Physiological effects of Ang II are thought to be mediated by specific high-affinity receptors present in Ang II-sensitive tissues including adrenal gland, vascular smooth muscle, uterus, myocardium, liver, pituitary, kidney and brain (reviewed in Mendelsohn, 1985; Capponi *et al.*, 1981). Although this receptor(s) has eluded genetic analysis, it has been characterized pharmacologically. In most tissues, Ang

II receptors demonstrate a single high affinity state with K_d values in the 0.1-5 nanomolar range. Occupation of Ang II receptors by Ang II induces two types of responses: stimulation of phosphatidyl inositol hydrolysis with subsequent calcium mobilization, and inhibition of adenylate cyclase. At one time, these dual responses seemed cell-specific. Ang II-stimulated cells from vascular smooth muscle, adrenal glomerulosa, renal mesangium, anterior pituitary and liver to induce phosphoinositide metabolism and calcium signalling, whereas Ang II action in uterine tissue inhibited adenylate cyclase. However, some tissues (vascular smooth muscle, pituitary and liver) have since demonstrated both responses. Such observations have evoked hypotheses of receptor subtypes analagous to the alpha adrenergic system (Garcia-Sainxz, 1987). Interestingly, recent studies using non-peptide Ang II antagonists have demonstrated the existence of pharmacologically distinct Ang II receptor subtypes (Whitebread *et al.*, 1989; Chiu *et al.*, 1989). In contrast, biochemical analysis of this receptor is still at an early stage. Preliminary evidence from covalent cross-linking and photoaffinity labeling studies indicate that the Ang II receptor consists of multiple subunits, each approximately 68,000 M_r (Paglin and Jamieson, 1982; Capponi *et al.*, 1980).

Ang II occupation of its receptor induces a variety of well-known effects in target tissues pertinent to regulation of blood pressure, fluid and electrolyte balance. Ang II

directly stimulates constriction of vascular smooth muscle as well as aldosterone biosynthesis in renal zona glomerulosa. In addition to these classical effects, recent investigation has extended the spectrum of Ang II activity in both circulating and tissue RAS. Ang II is now known to act upon the peripheral vasculature to stimulate prostaglandin release (Blumberg *et al.*, 1977) as well as angiogenesis (Fernandez *et al.*, 1985). Ang II has also been shown to increase myocardial contractility, stimulate pituitary hormone release and increase hepatic glucose metabolism (Mendelsohn, 1985). Thus, evidence suggests that Ang II is active in a variety of tissues and may demonstrate tissue-specific roles different from regulation of blood pressure and fluid balance.

Evidence For a Distinct Renin-Angiotensin System in the CNS

The presence of a distinct brain RAS has been firmly established (reviewed in Unger *et al.*, 1988). Interest in this sytstem began with the observation that centrally-injected Ang II caused profound increases in systemic blood pressure (Bickerton and Buckley, 1961). Subsequent studies also demonstrated the presence of Ang II in brain areas important to central control of blood pressure (Printz *et al.*, 1982). These observations, coupled with those indicating overactivity of this system in the spontaneously hypertensive rat (SHR) have led to the hypothesis that perturbations in brain RAS may play a role in essential

hypertension (Phillips, 1983). This hypothesis is perhaps best supported by observations that intracerebroventricular administration of captopril, an angiotensin converting enzyme (ACE) inhibitor, to SHR rats lowers blood pressure (Okuno *et al.*, 1983).

Although circumstantial evidence for a distinct brain RAS had existed for years (Phillips, *et al.*, 1979), only within the past decade have biochemical and molecular genetic studies conclusively demonstrated that brain possesses all components necessary to autonomously generate angiotensin peptides, including mRNA for angiotensinogen and renin (Ganten *et al.*, 1984). Pharmacologic studies have further demonstrated distinct receptors for Ang II in brain (Mendelsohn *et al.*, 1984). These observations have proven critical to acceptance of a distinct brain RAS since, in most areas of brain, this system is effectively isolated from the systemic RAS by the blood-brain barrier.

The *sine qua non* of an intrinsic RAS in brain, as in any tissue, is demonstration of local synthesis of angiotensinogen. Indirect evidence for the presence of this peptide in brain came from studies showing that Ang I could be recovered from brain extracts incubated in the presence of renin (Ganten *et al.*, 1971). More direct evidence was provided by reports of cell-free translations of brain mRNA yielding angiotensinogen identical to that of liver (Campbell and Habener, 1986). However, conclusive evidence for a brain source of angiotensinogen was provided only by direct *in situ*

hybridization studies of angiotensinogen mRNA in brain (Ganten *et al.*, 1984). Although brain-derived angiotensinogen is now accepted, the cellular source of this peptide is still controversial. Initial studies indicated the predominant site of angiotensinogen to be astrocytes and ependymal cells (Deschepper *et al.*, 1986). However, neuronal localization of this peptide has also been reported (Imboden *et al.*, 1987).

Although renin is considered the primary enzyme for angiotensinogen processing in the periphery, considerable debate centers on its participation in the brain RAS (Unger *et al.*, 1988; Printz, 1988). Recent evidence, including demonstration of renin mRNA in brain (Paul *et al.*, 1988), localization of renin in synaptosomal fractions of brain (Paul *et al.*, 1985) and inhibition of brain renin by specific renin antibodies (Ganten *et al.*, 1984), suggest that brain renin is "true renin" (Unger *et al.*, 1988).

Substantial evidence indicates that brain is an abundant source of converting enzyme (CE) which may explain the efficacy of ACE inhibitors in essential hypertension (Phillips, 1983). This enzyme is found in numerous brain structures including the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), basal ganglia, neurosecretory nuclei of the hypothalamus, median eminance, and posterior pituitary (Unger *et al.*, 1988). CE is found in ventricular choroid plexus, the ependyma, as well as in cerebrospinal fluid (CSF) (Rix *et al.*, 1981; Schelling *et*

al., 1980). Investigation into the cellular source of CE indicates that cultured neurons show higher CE activity than glia (Koshiya *et al.*,1985) and that CE, like renin, is localized to synaptosomal fractions of brain tissue (Paul *et al.*,1985)

The existence of endogenous angiotensin peptides has been suggested by both immunohistochemical localization of these peptides as well as pharmacological demonstration of their receptors in brain. Studies using high performance liquid chromatography combined with radioimmunoassay have provided direct biochemical evidence for the presence of Ang I and II in brain (Ganten *et al.*, 1983; Phillips and Stenstrom, 1985). Angiotensin II immunoreactivity has been demonstrated in hypothalamus, limbic system, medulla oblongata and spinal cord. Ang II-positive nerve terminals are found in high density in the median eminance, the paraventricular and supraoptic nuclei, as well as the SFO (Unger *et al.*, 1988). Central angiotensin synthesis has also been demonstrated in cultured brain tissue in the absence of peripheral RAS. In brain cultures, immunoreactive Ang II has been described (Weyhenmeyer *et al.*, 1980) and confirmed by [^{35}S]-methionine incorporation experiments in which immunoprecipitable Ang II was recovered from metabolically labeled brain cell proteins (Raizada *et al.*, 1983). Although immunohistochemical analyses of brain indicate a neuronal source of Ang II, its presence in glia is not clear. Subsequent immunocytochemical and HPLC studies

have, however, demonstrated both Ang I and Ang II in primary cell cultures of both neurons and glia (Hermann *et al.*, 1988).

Specific, high affinity receptors for Ang II have also been demonstrated and localized in brain (Mann, 1982; Mendelsohn *et al.*, 1984). These receptors have been found in several brain areas including septum, midbrain, thalamus, hypothalamus, and medulla oblongata as well as the OVLT and SFO, areas involved in central cardiovascular control (Landas *et al.*, 1980; Mendelsohn *et al.*, 1984). Ang II receptor autoradiography and immunohistochemical staining of Ang II demonstrate good correlation between peptide and receptor distributions (Healy and Printz, 1984a, b). Receptors for Ang II have also been demonstrated in primary cultures of neurons (Raizada *et al.*, 1984) and glia (Raizada *et al.*, 1987). Ang II receptors in neuronal cultures modulate catecholamine metabolism and levels (Sumners and Raizada, 1986a; Sumners and Phillips, 1983a) and themselves are regulated by catecholamines acting via $alpha_1$-adrenergic receptors (Sumners and Raizada, 1984; Sumners *et al.*, 1986b), by protein kinase C agonists (Sumners *et al.*, 1987a) and mineralocorticoids (Wilson *et al.*, 1986).

Stimulation of CNS Ang II receptors elicits a complex pattern of behavioral, cardiovascular and endocrine responses. Pressor responses, increases in thirst and release of several pituitary hormones, including vasopressin, oxytocin, ACTH and leutinizing hormone are most consistently

demonstrated following central administration of Ang II (Lang *et al.*, 1983). Several studies indicate that the OVLT and SFO, located in the lamina terminalis of the forebrain, are two areas pertinent to central Ang II responses (Printz *et al.*, 1982). Interestingly, the OVLT and SFO are highly vascularized structures which, unlike most of the brain, are accessible to the systemic RAS due to the absence of an intact blood-brain barrier in this area. Evidence for the participation of these structures in central Ang II responses are that lesions of the SFO and OVLT significantly blunt central responses to intravenous and intracerebral injection of Ang II (Simpson, 1981; Buggy and Johnson, 1978). The OVLT, on the other hand, seems to be the target of CSF-borne Ang II since unlike the SFO, it is accessible to CSF in cerebral ventricles. Cellular mechanisms of central Ang II responses are incompletely understood but are thought to be due to direct excitation of Ang II sensitive neurons in these areas, leading to activation of specific pathways eminating from the lamina terminalis and traveling through the anterior hypothalamus to the brainstem (pressor and thirst responses) or to the neurohypophysis (neurosecretory response) (Hartle and Brody, 1984). Although central effects of Ang II have been attributed primarily to actions on adrenergic neurons (Sumners *et al.*, 1983b), glial mediated effects cannot be ruled out.

Ang II: A Potential Role in Cellular Growth and Development

Evidence now suggests that activity of Ang II may reach beyond control of circulatory homeostasis to regulate both cellular growth and differentiation in select peripheral tissues. Specific binding sites for the Ang II receptor antagonist, [^{125}I]sar^{1}, ile^{8}-Ang II, have been demonstrated in several fetal tissues including the adrenals, kidney, liver and smooth muscle of blood vessels of the E19 rat fetus and suggest a role for Ang II in fetal growth and development (Millan *et al.*, 1989). Ang II increases DNA synthesis, protein synthesis and cell size in cultured vascular smooth muscle cells (Campbell-Boswell and Robertson, 1981; Geisterfer *et al.*, 1988; Berk *et al.*, 1989). Ang II stimulates induction of the growth-associated proto-oncogenes c-fos and c-myc (Naftilan *et al.*, 1989a; Naftilan *et al.*, 1989b) and stimulates angiogenesis (Fernandez *et al.*, 1985). Further evidence for a role of Ang II in growth regulation is recent identification of the Ang II receptor as the product of the *mas* oncogene (Jackson *et al.*, 1988). These observations thus suggest that Ang II may play an integral role in growth and development of certain cell populations in addition to its classical role in circulatory homeostasis. These observations have also raised the question whether Ang

II may demonstrate unique roles in brain pertinent to growth, development and regulation of different CNS cell types.

Cell Culture as a Model for the Study of Cell-Cell Interactions in the CNS

Current understanding of peptide factors involved in CNS intercellular communication has been made possible by the purification of these peptides from extracts of whole brain. Early work characterizing these factors relied heavily upon bioassay and explant culture, techniques limited by their inability to detect responses of individual cell types and incompatibility with rigorous biochemical analyses. Immortalized neuronal and glial cell lines, pure populations of cells amenable to biochemical analysis, often demonstrate anomalous growth factor production and responses and are thus limited in their applicability to the study of CNS growth and differentition factors. In many cases, availability of primary cell cultures of relativly pure populations of astrocytes, neurons and oligodendocytes have not only allowed for cell-specific identification of several factors important to both neuronal survival and growth regulation of CNS glia, but have been invaluable in delineation of mechanisms of action of known peptides on CNS target cells.

Brain cell culture has also been an invaluable tool in dissection of the brain RAS. As has been discussed previously, Ang I and Ang II have been demonstrated in primary cell cultures of neurons and glia (Hermann *et al.*,

1988). Receptors for Ang II have been found in primary cultures of neurons (Raizada *et al.*, 1984) and glia (Raizada *et al.*, 1987), and their regulation by different agents demonstrated (Sumners and Raizada, 1984; Sumners *et al.*, 1986b; Sumners *et al.*, 1987a; Wilson *et al.*,1986). Several physiological effects of Ang II have been observed in cultured neurons including modulation of catecholamine metabolism and levels (Sumners and Raizada, 1986a; Sumners and Phillips, 1983). Correlation between these observations and those from animal models clearly suggest that brain cells in culture are useful in the delineation of the brain RAS.

Study Objectives

Investigation of protein secretion by CNS astrocytes has implications for understanding the role of these cells in intercellular communication in the CNS. As discussed above, we believe that astrocytes of the brain may influence growth and differentiation of multiple CNS cell types and mediate CNS cellular responses to known peptide growth factors via the synthesis and secretion of proteins. It is likely that such protein secretion by these cells is developmentally regulated, given the plasticity and multiplicity of function of astrocytes during postnatal CNS development. To test this hypothesis, we have established primary cultures of astrocytes from neonatal and 21-day rat brain and analyze

these cells for their synthesis and secretion of novel developmentally-regulated proteins.

Although Ang II receptors have been demonstrated on astrocytes from neonatal rat brain (Raizada *et al.*, 1987), the role of this peptide in astrocytes from mature brain has not been investigated. To understand cellular events following Ang II stimulation of astrocytes and to ascertain potential developmentally-regulated astrocyte sensitivity to this peptide, we have used neonatal and 21-day rat brain astrocyte cultures to analyze and compare Ang II receptors and biochemical responsiveness of these cells to Ang II. Furthermore, we have investigated Ang II-induced changes in astrocyte protein secretion to determine whether CNS responses to this peptide may be mediated by astrocyte-derived secretory proteins during development.

Specifically, the objectives of these studies were:

1. To establish primary astrocyte cultures from neonatal and 21-day rat brain and to identify and characterize developmentally-regulated proteins secreted from these cells.
2. To characterize Ang II receptors and Ang II-stimulated biochemical responses, including DNA and protein synthesis, in established neonatal and 21-day rat brain astrocyte cultures to address potential developmental changes in Ang II responsiveness in these cells.

3. To characterize Ang II-induced changes in *de novo* synthesis and secretion of proteins from cultured neonatal and 21-day rat brain astrocytes to better understand cellular mechanisms of Ang II in the developing brain.

CHAPTER 2
METHODS

Rat Brain Astrocyte Cell Culture

Cultures of developing rat brain astrocytes were prepared essentially as described previously (Clark *et al.*, 1988; Raizada *et al.*, 1987) and are outlined schematically in Figure 2-1. Brains from 1-day (n=8-10),7-day (n=5), 14-day (n=4), 21-day (n=2), or adult (n=2) Sprague-Dawley rats were aseptically removed from the cranium at the level of the medulla oblongata and placed in an isotonic saline solution (pH 7.4), containing streptomycin (100 μg/ml), penicillin (100 U/ml) and amphotericin B (Fungizone) (2.5 μg/ml). Pia mater and blood vessels were carefully removed and remaining brain tissue minced into 2-3 mm^3 pieces. Minced tissue was then mechanically disrupted by triturion through a pasteur pipette and enzymatically digested for 7-10 min at 37° C in 20 ml of an isotonic saline solution (pH 7.4), containing 0.25% (w/v) trypsin, with 8 μg/ml deoxyribonuclease I added during the last 5 min of this period. Trypsin digestion was terminated by the addition of 20 ml of Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (DMEM/FBS) and the mixture centrifuged at 1000 x g for 5 min. The resulting cell pellet was resuspended in DMEM/FBS to a

density of 1 x 10^5 cells/ml, filtered through sterile gauze to remove nondissociated tissue, and 10 ml of this suspension added to poly-L-lysine coated 100 mm polystyrene tissue culture dishes (Falcon). Following this dissociation procedure, greater than 90% of the cells recovered were viable as determined by trypan blue exclusion. Primary mixed brain cell cultures were maintained in a humidified incubator (5% CO_2-95% [v/v] air) at 37°C for 3 days (2 days for 14, 21-day and adult astrocytes) after which time the culture medium was replaced with fresh DMEM/FBS. After an additional 6-8 days in culture, cells were rinsed once in an isotonic saline solution, containing penicillin (100 U/ml) and amphotericin B (Fungizone) (2.5 μg/ml) and dispersed by the addition of 0.25% trypsin for 5 min and frequent agitation. Dissociated cells were collected, centrifuged at 1000 x g for 5 min, resuspended in DMEM/FBS and replated on 100 mm tissue culture dishes at a density of 6400 cells/cm^2. Transfer of cells in this manner resulted in a relatively pure population of astrocytes since neuronal cells do not survive trypsin treatment. Secondary astrocyte cultures were grown 14-18 days before use.

Neonatal Rat Brain Neuron Cell Culture

The procedure for removal of neonatal rat brains and preparation of dissociated mixed brain cells is identical to that described for preparation of neonatal rat brain

astrocytes. Following enzymatic and mechanical dissociation of minced brain tissue, dissociated brain cells were added to 30 ml of DMEM, containing 10% plasma-derived horse serum (DMEM/PDHS) and centrifuged at 1000 x g for 5 min. The resulting cell pellet was resuspended in DMEM/PDHS and remaining non-dissociated tissue removed by filtration through sterile gauze. Cells were centrifuged at 1000 x g, resuspended in DMEM/PDHS to a concentration of 1.5×10^6 cells/ml, and plated on poly-L-lysine coated tissue culture dishes. Brain cells were adherent to culture dishes within 1 hr after plating. Neurons were subsequently maintained in culture in a humiified incubator (5% CO_2-95% [v/v] air) at 37°C. After three days, culture medium was removed and 10 M cytosine arabinoside (1% [w/v] ARC) in DMEM/PDHS added to selectively kill rapidly dividing cells. On the fifth day in culture DMEM/PDHS, containing 1% (w/v) ARC was removed and replaced with either DMEM alone, or DMEM/PDHS, as indicated. Cultures prepared by this method contained approximately 85% neurons as demonstrated by light and fluorescence microscopy using neuron-specific immunocytochemical markers, with remaining cells in these cultures identified as astrocytes.

Immunocytochemical Characterization of Cultures

Subconfluent cultures from neonatal and 21-day rat brain glial cultures and neonatal rat brain neuronal cultures, grown in 35 mm tissue culture dishes, were fixed with

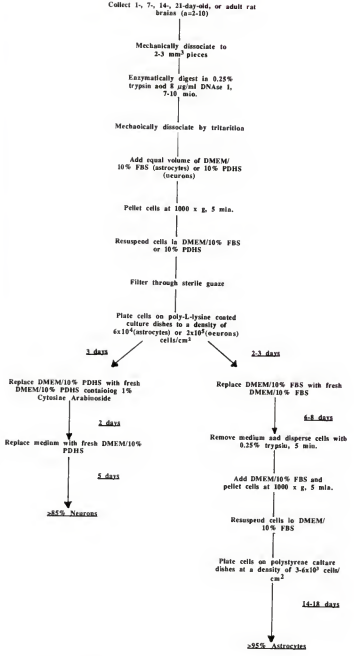

Figure 2-1. Summary of procedure for preparation of rat brain astrocyte and primary neuronal cell cultures.

phosphate-buffered saline (PBS), pH 7.4, containing 4% (w/v) paraformaldehyde, for 30 min at 4°C. Following four washes with PBS, cells were permeablized with 100% methanol for 5 min at -20°C. Permeablized cells were rinsed an additional four times with PBS and primary antibody (either rabbit anti-GFAP antisera [1:20 dilution in PBS containing 0.1% (w/v) sodium azide and 5% (w/v) nonfat dry milk] or mouse neurofilament-specific monoclonal antibody NE-14 [1:1000 in PBS]) added for 30 min at room temperature. Cells were washed five times in PBS and secondary antibody (either rhodamine-conjugated goat anti-rabbit immunoglobulin, 1:400 in PBS [for anti-GFAP], or fluorescein-conjugated goat anti-mouse immunoglobulin, 1:50 in PBS [for anti-NE-14]), added for an additional 30 min at room temperature. Cells were washed five times with PBS and coverslips applied with a 90% glycerol-10% PBS mounting medium. Fluorescent cells were then examined and photographed using a Nikon Axiophot™ microscope.

In vitro Metabolic Labeling of Cultures with L-[^{35}S]-methionine

Confluent neonatal and 21-day rat brain astrocyte cultures and ARC-treated neonatal rat brain neuron cultures were washed 3 times with serum-free MEM, containing 1/10th the original L-methionine concentration (10 μM), and incubated in this medium for 24 hr at 37°C. During the final 8 hr of the incubation period, cells were labeled with 100 μCi/dish L-

[^{35}S]-methionine. Cell viability, as determined by trypan blue exclusion, was greater than 97% following this incubation period. Cultures were terminated by collecting the culture medium and lysing the cells by the addition of 5 mM K_2CO_3 containing 9.4 M urea, 2% (w/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol. Cell lysates were scraped from the culture dishes, sonicated, centrifuged at 15,000 x g for 30 min at 18° C, and supernatants collected. Typically, less than 10% free [^{35}S]-methionine was present in the cell lysate samples, therefore dialysis was not performed. Medium samples were extensively dialysed (m.w. 3,500 cut-off) in 4 L of 10 mM Tris-HCl buffer, pH 8.3, containing 0.02% sodium azide, for 24 hr 4°C followed by 2 x 4 L changes, 12 hr each, of deionized water. All samples were stored at -20° C before analysis.

2D-SDS-PAGE

De novo synthesized proteins in cell lysate and conditioned medium samples from astrocyte and neuronal cultures were analyzed by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE) according to previously described methods (Roberts *et al.*, 1984). Briefly, aliquots of cell lysate and dialyzed conditioned medium samples were counted in a liquid scintillation counter to quantitate incorporation of [^{35}S]-methionine into non-dialyzable macromolecules. Equal amounts

(100,000 cpm) of radiolabeled protein in conditioned medium samples were then lyophilized and resuspended in 5 mM K_2CO_3 containing 9.4 M urea and 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol (DTT). Cell lysate samples did not require lyophilization and resuspension since less than 50 μl of sample was required to achieve 100,000 cpm of radiolabeled protein. [^{35}S]-methionine-labeled proteins from cell lysates and conditioned medium were separated by isoelectric focusing (75V for 0.5 hr, 150V for 2 hr, 300V for 15 hr followed by 450V for 1 hr) in 4% (w/v) polyacrylamide tube gels, containing 250 mM N,N'diallyltartardiamide, 9.4 M urea, 2% (v/v) Nonidet P-40, 0.5% (w/v) DTT and 5.1% (v/v) ampholytes (pH 3-10, 5-7 and 9-11; 50:36:16 by volume, respectively). Following isoelectric focusing, tube gels were kept at -20° C (typically less than 1 month) until further analysis. For separation in the second dimension, tube gels were thawed, equilibrated in 10-15 ml of 50 mM Tris-HCl, containing 1% SDS (w/v) and 1% (v/v) ß-mercaptoethanol for 15 min and applied to 10% polyacrylamide slab gels for electrophoresis (15 mAmp/gel for 2 hr followed by 25 mAmp/gel for 5 hr, constant current). Slab gels were fixed, stained in Coomassie Blue, destained, soaked in deionized water for 30 min followed by 1M sodium salicylate for 30 min and dried. Fluorograms were prepared by exposing dried gels to Kodak X-Omat R (XAR-5) for 7 days at -70° C.

Isolation and Sequencing of Astrocyte Secretory Proteins

To isolate pure isoelectric subunits for sequence analysis, 21-day rat brain astrocyte cultures in 100 mm culture dishes were extensively rinsed (3 x 4 ml) and maintained in serum-free MEM without [^{35}S]-methionine label for 24 hr at 37°C in a humidified incubator (5% CO_2-95% [v/v] air). Conditioned medium from 20-30 cultures (10 ml per dish) was collected and extensively dialyzed as described. Dialyzed 200-300 ml samples were lyophilized and resuspended and concentrated 100 times in 2-3 ml dH_2O. Typical protein concentrations, as determined by Bio-Rad protein assay, were 50-75 μg total protein/100 μl sample. Samples containing 250 μg protein were lyophilized, resuspended in 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40, and 5% (v/v) DTT, and subjected to 2D-SDS-PAGE followed by electrophoretic transfer for 15 hr in 25 mM Tris-base,pH 8.3, containing 192 mM glycine and 20% methanol to Immobilon™ membranes (Pluskal *et al.*, 1986). Following transfer, membranes were extensively rinsed 3x15 min in deionized water (this step removes free glycine and is critical to subsequent amino acid sequence analysis), stained with 0.1% Coomassie blue in 50% methanol, destained in 40% methanol-10% acetic acid (v/v), and rinsed with deionized water 3x5 min. Blots were stored at -20°C until sequencing.

N-terminal amino acid microsequencing of secreted proteins from 21-day rat brain astrocytes was performed at the University of Florida Protein Chemistry Core Facility using an Applied Biosystems Model 470A Gas Phase Protein Sequenator with an on-line analytical HPLC system. Since proteins of interest frequently appeared as closely-related groups by 2D-SDS-PAGE, two independent sequence analyses were usually made from spots of both acidic and basic regions of these groups in order to identify protein isoforms and to confirm sequence analysis.

Computer Analysis of Amino Acid Sequence Data

Amino acid sequence data were subsequently entered into the data base and analyzed using software from the Genetics Computer Group (GCG), version 6.0, made available on the VAX/VMS computer system of the University of Florida Institute of Food and Agricultural Sciences (IFAS) Computer Network. Sequence data entry and manipulation was performed using the SEQED interactive editing program. Peptide sequence secondary structure predictions, including estimations of antigenicity and surface probability, were generated using PEPTIDESTRUCTURE and displayed using PLOTSTRUCTURE protein analysis programs. Sequence data were compared to the National Biomedical Research Foundation (NBRF) and Genbank Genetic Sequence databases using FASTA and TFASTA programs (Pearson and Lipman, 1988) and match files

retrieved from these databases using the FETCH database file retrieval program.

Development and Testing of a Polyclonal Antiserum to an Astrocyte Secretory Protein

A polyclonal antiserum was generated to PVDF membrane-bound protein subunits by a modification of previously published methods (Chiles *et al.*, 1987). Briefly, secreted proteins from 100-fold concentrated culture medium from 21-day rat astrocytes were separated by 2D-SDS-PAGE, transferred to Immobilon™ PVDF membrane (Pluskal *et al.*, 1986), and visualized by Coomassie blue staining. Protein spots (2-3 μg protein per spot) of interest were excised from replicate blots (15-20) and stored at -20° C until used for immunization. Primary immunization was performed by implanting subcutaneously a total of 30-40 μg of PVDF membrane-bound protein into 5 sites along the back of a 1.8 kg male New Zealand white rabbit ("Dutch"). Each PVDF membrane-bound protein spot was moistened with Freund's complete adjuvant, rolled into the shape of a cylinder and inserted into the bore of a 16-gauge hypodermic needle. Immobilized protein was implanted in the rabbit by expelling it from the inserted needle with a stainless steel rod. Two secondary immunizations were similarly performed (30-40 μg/boost) and the rabbit bled from the central ear artery 14 days following each boost. Antibody production was assessed using immunoblots of proteins in unconcentrated 21-day rat

brain astrocyte conditioned medium separated by one-dimensional-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D-SDS-PAGE). Briefly, 1.0 ml of astrocyte conditioned medium was lyophilized, resuspended in 0.25 ml dH_2O and combined with an equal volume of 2X SDS sample buffer (1X sample buffer is 0.5M Tris-HCl, containing 2% [v/v] SDS, 10% [v/v] glycerol, 0.05% [w/v] DTT and 0.001% [w/v] bromophenol blue, pH 6.8). Samples were added to a single preparative well of a 7.5% stacking minigel, resolved through a 10% mini-gel and transfered to Immobilon™. Membranes were cut into 0.5 cm strips and analyzed by Western blotting (see following section for detailed methods) using serum from test bleeds. Antiserum collected 14 days after the second boost demonstrated the highest titer and was used for all subsequent experiments.

Western Blotting

Proteins in conditioned medium samples from cultures of astrocytes and neurons were subjected to 2D-SDS-PAGE as described, or discontinuous 1D-SDS-PAGE according to the method of Laemmli, (1970). For 1D-SDS-PAGE, reduced or nonreduced samples in sample buffer (0.5M Tris-HCl containing 2% [w/v] SDS, 10% [v/v] glycerol, 0.05% [w/v] and 0.001% [w/v] bromophenol blue, pH 6.8) were applied to 7.5% stacking gels and proteins resolved through a 10% gel at 15 mAmp/gel, constant current in a buffer containing 25 mM Tris-base, 192

mM glycine and 0.1% (w/v) SDS, pH 7. Following electrophoresis, gels were equilibrated in Towbin transfer buffer (25 mM Tris-base, 192 mM glycine, 20% [v/v] methanol, pH 8.3) for 10 min and proteins electroblotted onto nitrocellulose (0.45 μm, Bio-Rad, Richmond, CA) at 100 mAmp for 15 hr (Towbin *et al.*, 1979). Nitrocellulose was used as blotting substrate in these experiments since Immobilon™ nylon membranes gave higher nonspecific binding. Following transfer, nitrocellulose blots were rinsed with dH_2O (3 x 5 min), dried and then reequilibrated in 50 ml wash buffer (WB), containing 150 mM sodium chloride, 10 mM Tris-HCl, 0.2% (v/v) Triton X-100, 0.05% (w/v) sodium azide, pH 7.4, blocked with 2% (w/v) gelatin in WB for 1 hr and rinsed 2 x 5 min in 50 ml WB. Membranes were then incubated overnight at 4° C with antiserum diluted 1:500 in 25 ml WB containing 0.4% (w/v) heat-inactivated bovine serum albumin (BSA). Membranes were washed 4 x 5 min in 50 ml WB containing 2% (w/v) SDS, rinsed briefly in WB without SDS and incubated for 5 hr at 4° C with [^{125}I]-*staphylococcal aureas* protein A (1 x 10^6 cpm in 20 ml WB containing 0.4% BSA). Membranes were washed 4 x 5 min with WB, air-dried and bound radioactivity visualized by exposure to Kodak X-Omat AR film (Rochester, NY) for 2-8 days at -70° C.

Western Ligand Blotting

Proteins in samples of astrocyte and neuron conditioned medium were electrophoresed through discontinuous 10% polyacrylamide gels under nonreducing conditions and transferred to nitrocellulose membranes as described. Western ligand blotting was performed according to published methods (Hossenlopp *et al.*, 1986). Nitrocellulose membranes were rinsed 3 x 5 min with 50 ml Tris-buffered saline (TBS) (150 mM sodium chloride, 10 mM Tris-HCl, 0.05% (w/v) sodium azide), blocked with 25 ml 1% (w/v) non-fat dry milk for 1 hr and washed with TBS containing 0.1% Tween for 10 min. Membranes were incubated overnight at 4° C with 1 x 10^6 cpm [^{125}I]-IGF-2 in 5 ml TBS, containing 1% (w/v) BSA and 0.1% (v/v) Tween. Following incubation, membranes were rinsed briefly with 50 ml TBS, washed 2 x 15 min with 50 ml TBS, containing 0.1% (v/v) Tween and 3 x 15 min with 50 ml TBS. Membranes were air-dried and visualized by exposure to Kodak X-Omat AR film (Rochester, NY) for 18 hr at -70° C.

[^{125}I]-Angiotensin II Binding in Rat Brain Astrocyte Cultures

Specific binding of [^{125}I]-Ang II to cell membrane receptors was determined in confluent cultures of 21-day rat brain astrocytes according to published methods (Raizada *et al.*, 1987). Briefly, DMEM/FBS was removed from confluent cells in 35 mm tissue culture dishes and cells washed twice

with PBS. Triplicate cultures were incubated for up to 6 hr at 4° C (this temperature minimizes receptor-ligand internalization) with 0-2.5 nM [^{125}I]-Ang II and 0.8% (w/v) BSA in a final volume of 0.5 ml PBS, pH 7.2. Non-specific binding was similarly determined in triplicate dishes in the presence of 1 μM unlabeled Ang II. Following the incubation period, cultures were extensively rinsed (5 x 2 ml) with ice-cold PBS, pH 7.2, cellular protein dissolved in 0.5 ml 0.2 N NaOH and samples collected with the aid of a rubber policeman. Each plate was rinsed with 0.5 ml dH_2O which was combined with the original 0.5 ml sample. Radioactivity in each sample was determined in a Beckman 5500 gamma counter having a 75% efficiency for [^{125}I]. Specific binding of [^{125}I]-Ang II to astrocytes was calculated as the mean of triplicate samples, and was obtained by subtracting radioactivity bound in the presence of 1 μM unlabeled-Ang II from the total radioactivity bound. Saturation data were analyzed by the method of Scatchard, (1949).

[^{3}H]-Thymidine and [^{35}S]-Methionine Incorporaton in Rat Brain Astrocyte Cultures

Incorporation of [^{3}H]-thymidine by subconfluent cultures of neonatal and 21-day rat brain astrocytes was performed in culture dishes by a modification of previously described methods (Shemer *et al.*, 1987). DMEM/FBS was removed from subconfluent astrocyte cultures in 35 mm dishes and cells maintained an additional 24 hr in 2.0 ml serum-free DMEM at

37° C. Following 24 hr serum deprivation, cells were incubated with indicated concentrations of Ang II for an additional 24 hr at 37°C and cells pulsed with 1 μCi/dish [^{3}H]-thymidine during the last 1 hr of the incubation period. Incubations were terminated by removing culture medium and washing cells with 3 x 2 ml ice-cold PBS. Proteins and nucleic acids were precipitated by addition of 1.0 ml 10% (w/v) trichloroacetic acid (TCA) then 1.0 ml 5% TCA. Lipid-associated radioactivity was removed by 2 x 1 ml washes with ethanol:diethyl ether (3:1 [v/v], respectively). Precipitated macromolecules were solubilized in 0.5 ml 0.1 N NaOH overnight at 4° C, scraped from culture plates with a rubber policeman and collected. Culture dishes were washed with an additional 0.5 ml dH_2O which was then added to the original 0.5 ml cell lysate. Aliquots of these samples (250 μl) were combined with 10 ml Liquiscint® and counted in an LKB1217 Rack Beta Counter. Sample protein content was determined by the method of Lowry *et al.*, (1951)

Incorporation of [^{35}S]-methionine into cellular and secreted proteins from neonatal and 21-day rat brain astrocytes was determined as previously described. The only deviation from this procedure was that cell lysate samples were extensively dialyzed in 4 L dH_2O for 48 hr at 4° C to remove free [^{35}S]-methionine.

Densitometric Analysis of Fluorograms

Relative abundances of proteins visualized by 1D- or 2D-SDS-PAGE and fluorography were quantitated by densitometric analysis of fluorograms using a Zeineh Soft Laser Scanning Densitometer, Model SLR-TRFF. Bands or spots on developed films were scanned and analyzed using appropriate 1-dimensional or 2-dimensional scanning programs. Film exposure times were selected to produce neither overexposed nor underexposed bands, thus ensuring accurate analysis of flourograms. Since differences in apparent intensity of total labeled proteins often appeared among 2D-SDS-PAGE fluorograms within and between experiments (despite equal cpm loading and exposure to film), three control proteins (M_r 38,000, pI 6.6 (AGSP-38K); M_r 42000, pI 4.7; and M_r 63,400, pI 4.9) were scanned during analysis of each fluorogram and the sum of their densitometric values used to normalize fluorograms for total intensity differences. These three proteins were determined over the course of many experiments not to change in response to Ang II treatment and were thus considered appropriate controls for quantitation of Ang II experiments. Values from these analyses are expressed as relative densitometric units, normalized for differences in total fluorogram intensity

Preparation and Analysis of Total Cellular RNA from Astrocytes and Neurons

Extraction of total cellular RNA

Total cellular RNA from astrocytes and neurons in culture was isolated according to published methods (Chomczynski and Sacchi, 1987). Briefly, culture medium was removed from 100 mm culture dishes and cells rinsed briefly (3 x 5 ml) with PBS and lysed by addition of 2.0 ml/dish of 25mM sodium citrate, pH 7, containing 4M guanidinium isothiocyanate, 0.5% sarcosyl, and 0.1M ß-mercaptoethanol (solution D). Lysed cells were scraped from culture dishes, transferred to sterile centrifuge tubes, and RNA extracted by addition of 2M sodium acetate, phenol and chloroform-isoamyl alcohol (0.1 ml, 1.0 ml and 0.2 ml/ml solution D, respectively). RNA, present in the aqueous phase, was precipitated twice (at least 1 hr each) with equal volumes of isopropyl alcohol, washed once with 75% (v/v) ethanol and resuspended in 100 µl diethylpyrocarbonate (DEPC)-treated dH_2O. RNA purity and concentration were determined by the ratio of sample absorbance at 260 nm and 280 nm (1.8-2.0 for most preparations).

RNA gel electrophoresis

Isolated RNA (10-20 μg) was heated to 65° C for 20 min in sample buffer containing 0.02 M morpholinopropanesulfonic

acid, pH 7.0 (MOPS), 33% (v/v) formamide and 6% (v/v) formaldehyde, combined with tracking dye (0.25% [w/v] each, bromophenol blue and xylene cyanol in 50% [v/v] glycerol [2 µl/12 µl sample]) and ethidium bromide (0.2 µl of 10 mg/ml stock/12 µl sample) and chilled on ice. RNA was size-fractioned by electrophoresis at 70 V, constant voltage, with buffer circulation for 3-5 hr through horizontal agarose gels consisting of 1.2% (w/v) agarose, 0.02 M MOPS, 5 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA) and 6% (v/v) formaldehyde.

RNA blotting

Following electrophoresis, agarose gels were laid upside down on a wick made from 3 mm Whatman paper and covered with a GeneScreen™ nylon membrane, previously soaked 15 min in blotting buffer (12.5 mM NaH_2PO_4/12.5 mM Na_2HPO_4, containing 0.01% [w/v] sodium azide). RNA was passively tranferred overnight to GeneScreen™ with paper towels placed above the membrane to drive capillary flow. Following transfer, the blot was placed under ultraviolet light to visualize 18S and 28S ribosomal RNA bands, photographed and the negative used to ensure equal loading and transfer of samples. Blots were rinsed briefly in 2X SSPE (1X SSPE is 150 mM NaCl, 10 mM Na_2HPO_4 and 1 mM EDTA, pH 7.0), dried and baked for 2 hr at 80° C under vacuum to covalently bind RNA to the membrane

before hybridization. Membranes were stored dry in sealed bags at room temperature until needed.

Probes

Plasmids were obtained containing cDNA inserts for: rat IGF binding protein-2 (IGFBP-2) in pGEM3, from M.M. Rechler, Bethesda, MD and provided by F. Simmon, Gainesville, Fl, with permission; rat plasminogen activator inhibitor-1 (rPAI-1) in pBluescript kindly provided by T. Gelherter, Ann Arbor, MI; human tissue inhibitor of metalloproteases (hTIMP/TPA-S1), also in pBluescript, gift of I.B. Weinstein, New York, NY; and murine c-fos proto-oncogene in PBR322, purchased from American Type Culture Collection, Rockville, MD. Plasmid vectors containing a full cDNA clone for rPAI-1 (Zeheb and Gelehrter, 1988) and a genomic clone of c-fos (Van Beveren *et al.*, 1983) were amplified in E. Coli strains DH5 and MC1061, respectively, according to standard large scale preparative techniques (Maniatis *et al.*, 1989). The cDNA probe for PAI-1 will be used for experiments not presented in this dissertation. A plasmid vector (pGEM3) containing a 1.2 kb cDNA insert for IGFBP-2 (Brown *et al.*, 1989) was supplied in high quantitiy (500 µg) and did not require amplification prior to purification.

A 531 base pair (bp) SmaI-SacI fragment of the IGFBP-2 cDNA insert was excised from pGEM3 and gel-purified by the method of Benson, (198-). Briefly, 10 µg of plasmid DNA were

restriction endonuclease digested with 10 units each, Sst I (an isoschizmer of Sma I) and Sac I in appropriate restriction buffer (BRL buffer 4) containing 0.5%(w/v) BSA for 2 hr at 37° C. Reactions were stopped by the addition of 1 mM EDTA, combined with sample buffer (10% [v/v] glycerol and 0.5% [w/v] each, bromophenol blue and xylene cyanol) and fragments separated by electrophoresis through a 1.4% agarose gel, containing 1 µg/ml ethidium bromide, in 1X TBE (0.09M Tris-borate and 2.0 mM EDTA, pH 8.0). The 531 bp Sma I-SacI fragment was visualized under UV light, excised from the gel and extracted. Briefly, gel fragments containing DNA were pulverized by placement in a tuberculin syringe and rapid passage through a 23 guage needle into a 1.5 ml microfuge tube, combined with 100 µl distilled phenol, vortexed for 30 sec and frozen at -70° C in a dry ice-ethanol bath for 1 hr. Sample was then centrifuged for 15 min at room temperature. The resulting supernatant containing DNA was removed, extracted twice with equal volumes (approximately 300 µl) phenol and DNA precipitated by the addition of 5 M NaCl (15 µl/100 µl supernatant), 2.5 volumes ice-cold 95% ethanol and storage at -70° C for 1 hr. The sample was centrifuged, DNA pellet washed once with 1 ml ice-cold 95% ethanol and dried under vacuum. The final pellet was resuspended in 30 µl TE (10 mM Tris buffer, pH 8 1, containing 1 mM EDTA) and 3 µl of this solution run on a 1.4% agarose minigel with molecular size markers of known concentration (jX174 Hae III ladder) to

both confirm purity and estimate insert concentration (50 ng/ml).

Preparation of ^{32}P-labeled IGFBP-2 cDNA probe

The 531 bp probe for IGFBP-2 was labeled by the method of "random-primer extension" (Feinberg and Vogelstein, 1984) using a commercially available kit (Prime-it™, Stratagene). After heating to 95-100° C for 5 min insert DNA and random oligonucleotide (nonamer) primers were incubated with dATP, dGTP, dTTP (buffer composition and dNTP concentrations not revealed by manufacturer) and [^{32}P]dCTP (50 µCi, 6000 Ci/mmol) in the presence of 4 units T7 DNA polymerase for 10 min at 37° C. Labeling was terminated by the addition of stop mix (composition not revealed by manufacturer). [^{32}P]-labeled insert was purified over an ELUTIP column, prepared for use by exposure to 5 ml of "high-salt" buffer (1 M NaCl, 20 mM Tris, pH 7.4 and 1 mM EDTA), followed by 5 ml of "medium-salt" buffer (0.4 M NaCl, 20 mM Tris, pH 7.4 and 1 mM EDTA). The entire labeling reaction mixture (50 µl), combined with 480 µl "medium-salt" buffer, was passed over the column, the column washed with 3.5 ml "medium-salt " buffer to remove unincorporated label and [^{32}P]-labeled insert eluted with 0.5 ml "high salt" buffer. Following ethanol precipitation of [^{32}P]-labeled insert for 30 min at -70° C (facilitated by the addition of 1 µl tRNA [5 µg/µl in dH_2O]) and drying of the

pellet under vacuum, total radioactivity of the probe was determined using a Dupont BC2000 beta counter.

Entire pBR322 plasmids containing the 7.1 kb genomic clone of the murine c-fos gene were prepared by the method of nick translation (Maniatis *et al.*, 1989) using a commercially available kit (Nick Translation System, BRL). Briefly, plasmid containing insert (0.5 µg) was mixed with 10X Nick Translation Buffer C (0.2 mM dATP, dGTP, dTTP in 0.5 M Tris-HCl, pH 7.5, 0.1 M $MgSO_4$, 1 mM DTT, 500 µg/ml BSA) and 100 µCi [^{32}P]dCTP (6000 Ci/mmol, 10 µCi/µl), brought to a volume of 18 µl, and chilled on ice. DNAse I (1.5 µl of a 10 ng/ml stock) and 2.5 units E. Coli DNA polymerase I (0.5 µl of 5 unit/µl stock) were then added, the entire mixture vortexed and incubated for 1 hr at 16° C. The reaction was stopped by the addition of 2 µl 0.2 M EDTA and the [^{32}P]-labeled c-fos plasmid probe separated from unincorporated radioactivity using a pasteur pipette column containing TE-saturated Bio-Gel A-1.5m 100-200 mesh beads (Bio-Rad). Incorporated radioactivity was quantitated by liquid scintillation methods and was determined to be 4.4 x 10^8 cpm/µg DNA.

Northern Hybridization

Hybridization of [^{32}P]-labeled probes to membrane-bound RNA was performed by two methods. For detection of IGFBP-2 mRNA, nylon membranes were incubated in 7.5 ml prehybridization solution (10% [w/v] dextran sulfate, 50%

[v/v] formamide, 5X SSPE, 0.1% [w/v] Denhardt's solution [1% Denhardt's is 1% (w/v) each, Ficoll, polyvinylpyrrolidone and BSA], 1% [w/v] SDS and 100 µg/ml heat-denatured salmon sperm DNA) for 1.5 hr at 50° C, while a second nylon membrane (without RNA) was similarly incubated in 7.5 ml hybridization solution (pre-hybridization solution plus probe, 5 x 10^5 cpm total). Following this prehybridization period, 7.5 ml of prehybridization mixture containing [^{32}P]-labeled IGFBP-2 probe was removed from the blank membrane and added to the bag containing the RNA-bound membrane, and hybridization was continued for 24 hr at 50° C. Hybridized blots were washed 2 x 30 min at 50° C with 250 ml of pre-warmed low stringency wash buffer (2X SSPE, 0.2% SDS) followed by 1 x 15 min wash and 1 x 5 min wash at room temperature with pre-warmed (60° C) high stringency wash buffer (0.1X SSPE). Membranes were allowed to dry and bound radioactivity determined using a Betagen™ radiodensitometric scanner. Autoradiograms were also obtained by exposing blots to Kodak X-Omat R (XAR-5) film for 18-24 hr at -70° C.

Detection of c-fos mRNA was performed by prehybridizing blots in 20 ml 6X SSPE, pH7.4, containing 50% (v/v) formamide, 0.1% (w/v) Denhardt's solution, 0.5% (w/v) SDS and 100 µg/ml heat-denatured salmon sperm for 4 hr at 42° C Blots were hybridized for 24 hr at 42° C in an identical solution supplemented with 10% (w/v) dextran sulfate and [^{32}P]-labeled probe. Following hybridization, membranes were washed 2 x 5 min in 250 ml 2X SSC (20X SSC is 3 M NaCl, 0.3M

Na citrate, pH 7.0), containing 0.2% SDS at room temperature and 3 x 30 min in 150 ml 0.1X SSC containing 0.1% SDS at 52° C. Membranes were allowed to dry and bound radioactivity determined using a Betagen™ radiodensitometric scanner. Autoradiograms were also obtained by exposing blots to Kodak X-Omat (XAR-5) film for 18-24 hr at -70° C.

Statistical Analysis

Where mean experimental values between two groups were compared, Student's unpaired t-test was used with $p < 0.05$ as the minimum criterion for significance.

CHAPTER 3
CHARACTERIZATION AND IDENTIFICATION OF A DEVELOPMENTALLY-REGULATED ASTROCYTE SECRETORY PROTEIN

Introduction

Astrocytes of the mammalian CNS, long considered passive support elements for neurons, are now recognized as active participants in development and maintenance of normal brain physiology. A growing body of evidence indicates that these cells and their precursors may play crucial roles in several key developmental processes in the brain including neuronal migration (McKay, 1989), axonal sprouting and guidance (Hatten and Mason, 1986), neuroglial growth and differentiation (Raff, 1989), neurotransmitter metabolism (Hertz and Schousboe, 1986) and induction of the blood-brain barrier (Janzer and Raff, 1987).

Growth, differentiation and maintenance of CNS neurons, oligodendrocytes and astrocytes are thought to depend upon close biochemical associations between these cells of the brain. Although well-established anatomical relationships among neurons, oligodendrocytes and astrocytes have suggested interactive processes between these cells, knowledge of glial-neuronal and glial-glial interactions is indeed limited. Furthermore, factors which may mediate such interaction in developing brain have remained poorly defined.

In recent years, however, considerable progress has been made in this area by several studies which describe the presence of neurotrophic as well as glial regulatory factors in the brain. Well-characterized neurotrophic factors include nerve growth factor (NGF) (Cohen, 1960; Thoenen and Edgar, 1985), brain-derived growth factor (BDNF) (Barde *et al.*, 1982), fibroblast growth factors (aFGF, bFGF) (Walicke *et al.*, 1988), glial-derived protease nexin I (GdPN-1) (Geunther *et al.*, 1985) and the insulin-like growth factors (IGF 1 and 2) (Recio-Pinto and Ishii, 1984). Several glial regulatory factors have been characterized and include plasma-derived growth factor (PDGF) (Raff *et al.*, 1988), interleukin 1 (IL-1) (Giulian *et al.*, 1988b), GdPN-1 (Gloor *et al.*, 1986), epidermal growth factor (EGF) (Wang *et al.*, 1989), and IGF-1 (Shemer *et al.*, 1987).

Evidence now suggests that CNS astrocytes may be an important source of these and other neurotrophic and glial-regulatory peptides. Neurons cultured in the presence of astrocytes "fare much better" than in their absence and astrocyte conditioned medium has been shown to mimic this trophic effect (Banker, 1980). Astrocyte-derived C6 glioma cells secrete over four hundred *de novo* synthesized proteins into culture medium (Arrenander and de Vellis, 1980; Arrenander and de Vellis, 1981) and at least seven of these, including NGF, have demonstrated neurotrophic properties (Westermann *et al.*, 1988). Astrocytes have been shown to also secrete a peptide immunologically related to PDGF which

drives oligodendrocyte differentiation (Raff *et al.*, 1988). Finally, astrocytes may control their own proliferation by secretion of autocrine growth regulatory peptides. Several unidentified glial-inhibitory peptides have been found in conditioned medium from C6 glioma cells (Aloisi *et al.*, 1987).and GdPN-1, a neurotrophic as well as glial regulatory factor, is produced by astrocytes in primary culture (Schurch-Rathgeb and Monard, 1978).

Developmental plasticity of the CNS likely dictates age-dependent changes in astrocyte cellular physiology. Astrocytes cultured from fetal and neonatal rat brain differentially express subtypes of muscarinic cholinergic receptors, only one of which mediates carbachol-induced increases in DNA synthesis in these cells (Ashkenazi *et al.*, 1989). During development, nonneuronal cells differentially synthesize and secrete apolipoprotein E, a M_r 37,000 lipid binding protein involved in axonal myelination (Skene and Shooter, 1983; Muller *et al.*, 1986). These observations suggest that age-dependent changes in production of soluble peptide factors by astrocytes may mediate dynamic neuronal-glial and glial-glial interactions in the developing brain.

To test the hypothesis that astrocytic glial cells demonstrate selective developmental changes in their synthesis and secretion of proteins, we have established primary astrocytic glial cultures from brains of neonatal, 7-day, 14-day, 21-day, and adult rats and have employed two-dimensional sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (2D-SDS-PAGE) to determine *de novo* synthesized proteins in culture medium conditioned by these cells. We propose that identification of unique developmentally-regulated proteins may give insight into glial-neuronal and glial-glial interactions in the developing rat CNS.

Results

Immunocytochemical Characterization of Cultures

Cultures of neonatal and 21-day rat brain astrocytes and neonatal rat brain neurons were characterized morphologically as well as for expression of astrocyte- and neuron-specific immunologic markers using standard techniques (Figure 3-1). Phase contrast examination of glial cultures from neonatal and 21-day rat brain reveals that both cultures contain flat polygonal cells tightly adhered to culture dishes. Immunofluorescent staining of these cells with an antiserum against glial fibrillary acidic protein (GFAP) followed by quantitation and comparison of GFAP-positive cells in both neonatal and 21-day rat brain cultures indicates that 95-98% of cells in each culture are astrocytes. These cultures are also comparable with respect to number of cells (1×10^6) and protein content (130-180 µg) per 35 mm culture dish. Phase contrast examination of neuronal cultures from neonatal rat brain shows cells with neurites extending from discrete cell

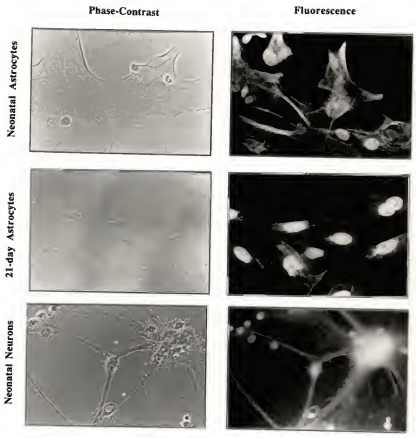

Figure 3-1. Phase contrast and fluorescence microscopic identification of neonatal and 21-day rat brain astrocytes and neonatal rat brain neurons in primary culture. Subconfluent glial cultures from neonatal (top) and 21-day rat brain (middle) were incubated with a rabbit polyclonal antiserum to glial fibrillary acidic protein (GFAP) followed by a rhodamine-conjugated goat anti-rabbit secondary antiserum as described in *Methods*. Neuronal cultures from neonatal rat brain were similarly treated using a monoclonal primary antibody (NE14) to neurofilaments followed by a fluorescein-conjugated goat anti-mouse secondary antiserum. Immunostained cultures were visualized by phase contrast (left) and fluorescence microscopy (right).

bodies, thus demonstrating typical neuronal morphology. These cells overlie larger polygonal cells identified as mostly type I astrocytes which represent up to 15% of total cells in these neuronal cultures (Sumners *et al.*, 1990). Immunofluorescent staining of these cultures with anti-neurofilament monoclonal antibodies (NE-14) clearly identifies neurofilaments within neurites.

Analysis of [^{35}S]-methionine-labeled cellular and secretory proteins from neonatal and 21-day rat brain astrocytes by 2D-SDS-PAGE

Representative 2D-SDS-PAGE profiles of [^{35}S]-methionine-labeled cellular and secretory proteins from neonatal and 21-day rat brain astrocytes are shown in Figure 3-2. Comparison of profiles of [^{35}S]-methionine-labeled cellular proteins from neonatal and 21-day rat brain astrocytes indicates that these cells are very similar in their constitutive production of cellular proteins. A notable exception is a basic protein, M_r 27,000, pI 7.6 preferentially produced by 21-day rat brain astrocytes (arrowhead). The major [^{35}S]-methionine-labeled secretory proteins from both cultures include three groups: 1) M_r 39,000, pI 5.0-5.4; 2) M_r 46,000, pI 4.5-4.8; and 3) M_r 47,000, pI 4.8-5.0. In contrast to cellular proteins, striking differences were observed in *de novo* synthesis and secretion of proteins by astrocytes from the two age groups. Neonatal rat brain astrocytes secreted a group of proteins

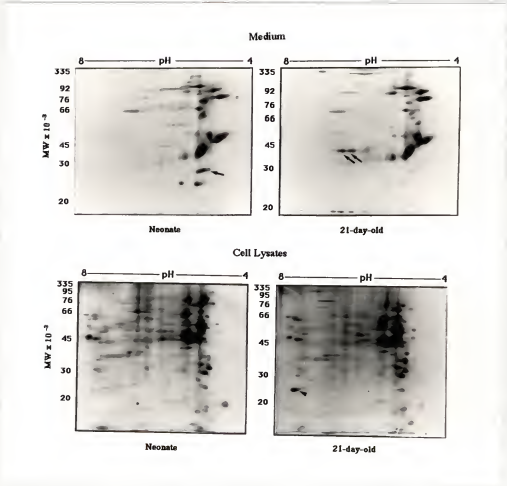

Figure 3-2. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled cellular and secretory proteins from neonatal and 21-day rat brain astrocytes. Astrocytes were extensively rinsed in serum-free MEM containing 10 μM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C. During the final 8 hr of the incubation period, cells were pulsed with 100μCi/dish L-[^{35}S]-methionine. Cultures were terminated and conditioned medium and cell lysate samples processed and analysed by 2D-SDS-PAGE followed by fluorography as described in *Methods*. Arrowhead indicates a M_r 27,000 basic cellular protein produced by 21-day rat brain astrocytes. Single arrow indicates a M_r 28,000 acidic protein secreted by neonatal rat brain astrocytes. Double arrow indicates a M_r 35,000 protein secreted by 21-day rat brain astrocytes.

(2-3 isoforms), M_r 28,000, pI 4.7-5.5 (NGSP-28K) (arrow), while astrocytes from 21-day rat brain selectively secreted a distinct family (four isoforms) of proteins, M_r 35,000, pI 6.3-6.8 (AGSP-35K) (double arrow). Treatment of neonatal and 21-day astrocyte cultures with 1.0 μg/ml tunicamycin (Figure 3-3) indicated that *de novo* synthesis and secretion of NGSP-28K from neonatal astrocytes was sensitive to inhibition of N-linked glycosylation, whereas synthesis and secretion of AGSP-35K by 21-day rat brain astrocytes was not affected.

Analysis of CNS cell-specificity and developmental regulation of AGSP-35K secretion using a rabbit polyclonal antiserum raised against purified AGSP-35K

To accurately address cell specificity and developmental regulation of AGSP-35K secretion by cultured CNS astrocytes, a rabbit polyclonal antiserum was generated to purified AGSP-35K according to the procedure described in *Methods*. Analysis of Western blots of 21-day rat brain astrocyte secretory proteins which were separated by 2D-SDS-PAGE demonstrated that this antiserum specifically detects AGSP-35K (Figure 3-4, arrows). Nonspecific staining at approximately M_r 60,000, pI 4-8 was observed in blots tested with both anti-AGSP-35K and preimmune serum (Figure 3-4). This nonspecific background staining has been reported byother investigators using many different antisera (unpublished).

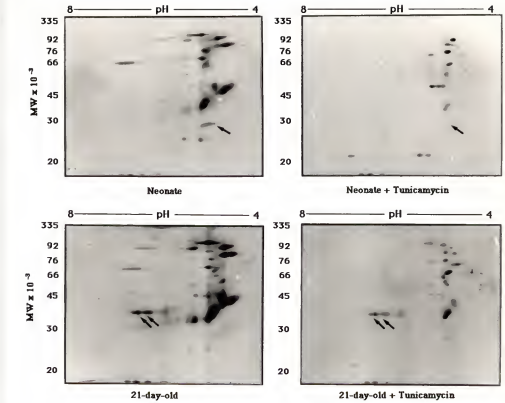

Figure 3-3. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled cellular and secretory proteins from neonatal and 21-day rat brain astrocytes treated with tunicamycin. Astrocytes were extensively rinsed in serum-free MEM containing 10 µM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C in the presence of 1 µg/ml tunicamycin (dissolved in DMSO) or DMSO alone. During the final 8 hr of the incubation period, cells were pulsed with 100µCi/dish L-[^{35}S]-methionine. Cultures were terminated and conditioned medium and cell lysate samples processed and analysed by 2D-SDS-PAGE followed by fluorography as described in *Methods*. Double arrow indicates a M_r 35,000 protein secreted by 21-day rat brain astrocytes in the absence and presence of 1 µg/ml tunicamycin.

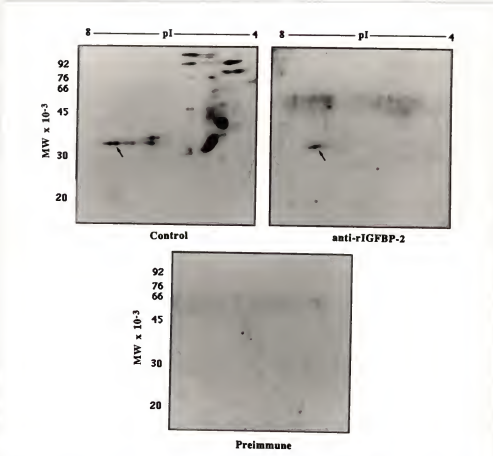

Figure 3-4. Representative fluorogram and immunoblots obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled secretory proteins from 21-day rat brain astrocytes. [^{35}S]-methionine-labeled secretory proteins in conditioned culture from 21-day rat brain astrocytes were subjected to 2D-SDS-PAGE followed by either fluorography or immunoblotting with an antiserum raised against AGSP-35K as described in *Methods*. *Top, left*. Fluorogram of total [^{35}S]-methionine-labeled secretory proteins from 21-day rat brain astrocytes. Arrow indicates radiolabeled AGSP-35K protein preferentially secreted by 21-day rat brain astrocytes. *Top, right*. Immunoblot of secretory proteins with anti-AGSP-35K (1:500). Arrow indicates AGSP-35K specifically recognized by the antiserum. Bottom. Immunoblot of secreted proteins with preimmune serum (1:500). *Note*: Non-specific binding at ~M_r 60,000, pI 4-8 is present in blots tested with both anti-AGSP-35K and preimmune serum.

To investigate CNS cell specificity of AGSP-35K secretion, cell lysate and conditioned medium proteins from neonatal rat brain astrocytes, 21-day rat brain astrocytes and neonatal rat brain neurons were separated by one-dimensional (1D-) SDS-PAGE under reducing conditions and blotted. When blotted samples were exposed to anti-AGSP-35K antiserum (Figure 3-5), three major bands were detected in 21-day rat astrocyte conditioned medium (lane 4): one band, M_r 32,000, and two bands M_r <10,000. Conditioned medium from neonatal rat brain astrocytes (lane 2) showed a faint M_r 32,000 band whereas no specific IGFBP-2 immunoreactivity was detected in conditioned medium from neonatal rat brain neurons. Faint immunoreactive bands with slightly slower electrophoretic mobility than M_r 32,000 were detected in cell lysates from all three cell types (lanes 1, 3 and 5) and may represent a cross-reactive protein or pre-formed AGSP-35K precursor. Testing of 21-day astrocyte cell lysate and medium samples (lanes 7 and 8) with pre-immune serum indicated that all other bands were nonspecific in nature. Differences in electrophoretic mobility of AGSP-35K observed in 1D versus 2D-SDS-PAGE (M_r 32,000 *vs* 35,000) may be due to differences in gel reagents, running conditions, or more likely, differences in mobility of pre-stained versus unstained molecular weight standards used in 1D- and 2D-SDS-PAGE, respectively.

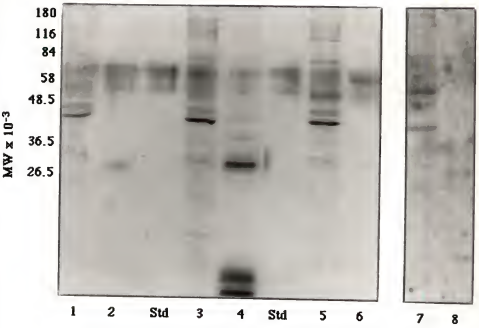

Figure 3-5. Comparison of AGSP-35K secretion by neonatal and 21-day rat brain astrocytes and neonatal rat brain neurons by Western blotting with anti-AGSP-35K. Proteins in cell lysate and conditioned medium samples from neonatal and 21-day rat brain astrocyte and neonatal rat brain neurons were separated by one-dimensional SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described in *Methods*. *Lane1*, Cell lysates from neonatal astrocytes. *Lane 2*, Conditioned medium from neonatal astrocytes. *Lane 3*, Cell lysates from 21-day astrocytes. *Lane 4*, Conditioned medium from 21-day astrocytes. *Lane 5*, Cell lysates from neonatal neurons. *Lane 6*, Conditioned medium from neonatal neurons. *Lanes 7 and 8*, Duplicate samples from lanes 3 and 4, respectively, tested with pre-immune serum. *Std*, Molecular weight standard lanes. Protein content in each lane is equivalent to 100,000 cpm of labeled proteins analyzed by 2D-SDS-PAGE. Arrow indicates position of AGSP-35K. M_r 46,000 bands in cell lysate samples and ~M_r 60,000 bands in all samples were equally detected with preimmune serum.

Developmental regulation of AGSP-35K secretion by rat brain astrocytes was determined in primary cultures derived from neonatal, 7-day, 14-day, 21-day, and adult (95-day) rats. Proteins in conditioned medium from these astrocytes were separated by 1D-SDS-PAGE, blotted, and tested with anti-AGSP-35K. As shown in Figure 3-6, astrocytes cultured from all five age groups secrete detectable levels of AGSP-35K. However, synthesis and secretion of AGSP-35K increased significantly in rat brain astrocytes cultured between postnatal days 7 and 14, and remained high in astrocytes cultured from 21-day and adult animals.

N-terminal amino acid sequence determination of AGSP-35K and identification of this protein as the BRL-3A insulin-like growth factor binding protein, IGFBP-2

To better understand the significance of expression of NGSP-28K by neonatal rat brain astrocytes and AGSP-35K by 21-day rat brain astrocytes, medium from these cells was concentrated, proteins separated by 2D-SDS-PAGE and transferred to Immobilon™ PVDF membranes, and N-terminal amino acid sequencing performed by automated gas-phase amino acid microsequencing. Using this technique, 29 of the first 33 N-terminal amino acids from AGSP-35K were conclusively identified and are shown in Figure 3-7. Four cysteine residues were tentatively identified but were unable to be positively determined. In addition, identical amino acid sequences were obtained from basic (pI 6.8) and acidic

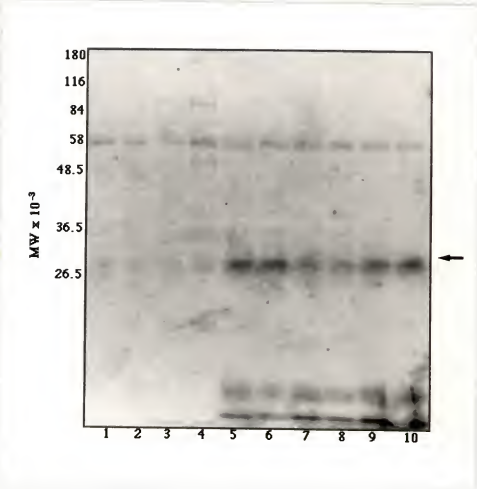

Figure 3-6. Comparison of AGSP-35K secretion by neonatal, 7-day, 14-day, 21-day and adult rat brain astrocytes by Western blotting with anti-AGSP-35K. Proteins in conditioned medium samples from two cultures each of neonatal, 7-day, 14-day, 21-day and adult rat brain astrocytes were separated by 1D-SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described in *Methods*. *Lanes 1 and 2,* neonatal astrocytes. *Lanes 3 and 4,* 7-day astrocytes. *Lanes 5 and 6,* 14-day astrocytes. *Lanes 7 and 8,* 21-day astrocytes. *Lanes 9 and 10,* adult astrocytes. Protein content in each lane is equivalent to 100,000 cpm of labeled proteins analyzed by 2D-SDS-PAGE. Arrow indicates position of AGSP-35K. ~M_r 60,000 bands in all samples were equally detected with preimmune serum.

```
                10                  20                  30
AGSP-35K  EVLFRCPPCT PERLAACGPP PDAPCAELVR EPG
          :::::::::: :::::::::: :::::::::: :::
rIGFBP-2  EVLFRCPPCT PERLAACGPP PDAPCAELVR EPG
                10                  20                  30
```

Figure 3-7. N-terminal amino acid sequence of AGSP-35K and comparison to the BRL-3A insulin-like growth factor binding protein, rIGFBP-2. Secreted proteins in conditioned medium from cultures of 21-day rat brain astrocytes were concentrated, resolved by 2D-SDS-PAGE and transfered to Immobilon™ membranes as described in *Methods*.. N-terminal amino acid microsequencing of AGSP-35K was performed and sequence data compared to database entries using the FASTA and TFASTA programs of Pearson and Lipmann. *Top,* N-terminal sequence comparison of amino acids 1-33 of AGSP-35K to amino acids 1-35 of the mature rIGFBP-2 (Brown *et al.*, 1989) indicates 100% sequence identity (indicated by :) between these two regions.

(pI 6.3) regions of this family suggesting that variation in pI values between individual spots within this group may represent different degrees of post-translational modification of a single protein. Sequence analysis of the neonatal astrocyte secretory protein, NGSP-28K was unsuccessful due to the presence of a modified N-terminal amino acid on this protein.

Sequence data from AGSP-35K were compared to reported NBRF and Genbank sequences using both the FASTA and TFASTA database search programs. As shown in Figure 3-7, these 33 amino acids demonstrate 100% identity to the same region of rat insulin-like growth factor binding protein, rIGFBR-2, a M_r 30-35,000 non-glycosylated protein previously characterized in rat BRL-3A fetal liver cell line (Brown *et al.*, 1989; Yang *et al.*, 1990).

Analysis of IGFBP-2 expression in astrocytes and neurons by Western and Western Ligand blotting

Although molecular weight and sequence data strongly suggested that AGSP-35K was astrocyte-derived IGFBP-2, confirmation of this assignment required demonstration of IGF binding. Western and Western Ligand blots of cell lysates conditioned medium from rat brain astrocytes and neurons using anti-AGSP-35K antiserum and [^{125}I]-IGF-2 is shown in Figure 3-8. When nonreduced cell lysate and conditioned medium samples identical to those analyzed in Figure 3-7 were tested by Western ligand blotting with [^{125}I]-IGF-2 (Figure 3-

8) one major band (lane 4), M_r 28,000 and one minor band M_r < 10,000 were detected in 21-day rat astrocyte conditioned medium. Conditioned medium from neonatal rat brain astrocytes (lane 2) demonstrated two faint bands: one, M_r 28,000 and another, M_r 35-40,000. [^{125}I]-IGF-2 binding was not detectable in conditioned medium from neonatal rat brain neurons (lane 6), nor in cell lysates from the three cell types (lanes 1, 3 and 5). A Western blot of nonreduced 21-day rat astrocyte conditioned medium using anti-AGSP-35K antiserum (Figure 3-8, lane 7) detected two bands: one, M_r 28,000 and another, M_r <10,000 indicating that apparent differences in electrophoretic mobility between samples analyzed by Western and Western ligand blots were due to reducing conditions. These results support the assignment of AGSP-35K as IGFBP-2 and further demonstrate increased synthesis and secretion of this protein by 21-day rat brain astrocytes compared to astrocytes from the neonate.

Demonstration of IGFBP-2 mRNA in astrocytes from neonatal and 21-day rat brain

Protein data from 2D-SDS-PAGE, Western, and Western ligand analyses of astrocyte conditioned medium indicate that 21-day rat brain astrocytes synthesize and secrete high levels of IGFBP-2 compared to neonatal rat brain astrocytes and that neonatal rat brain neurons do not secrete this protein. Northern analysis of total cellular RNA from these three cell types using a purified IGFBP-2 cDNA insert was

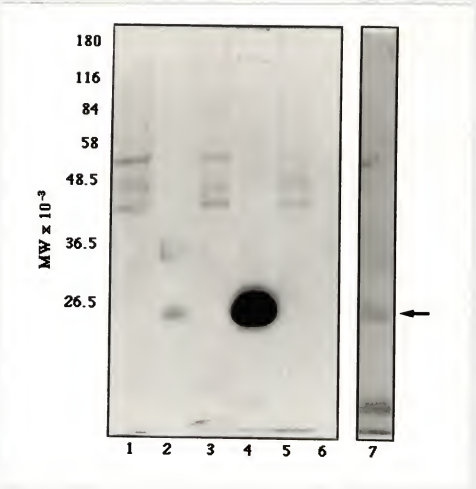

Figure 3-8. Comparison of IGF binding protein secretion by neonatal and 21-day rat brain astrocytes and neonatal rat brain neurons by Western ligand blotting with [^{125}I]-IGF-2 and by Western blotting with anti-AGSP-35K/rIGFBP-2. Proteins in cell lysate and conditioned medium samples from neonatal and 21-day rat brain astrocyte and neonatal rat brain neurons were separated by 1D-SDS-PAGE under nonreducing conditions, transferred to nitrocellulose membranes, and ligand blotted as described in *Methods* (lanes 1-6). A duplicate medium sample from 21-day astrocytes was run separately under nonreducing conditions and tested with anti-AGSP-35K (lane 7). *Lane 1*, Cell lysates from neonatal astrocytes. *Lane 2*, Conditioned medium from neonatal astrocytes. *Lane 3*, Cell lysates from 21-day astrocytes. *Lane 4*, Conditioned medium from 21-day astrocytes. *Lane 5*, Cell lysates from neonatal neurons. *Lane 6*, Conditioned medium from neonatal neurons. *Std*, molecular weight standard lanes. *Lane 7*, Conditioned medium from 21-day astrocytes. Protein content in each lane is equivalent to 100,000 cpm of labeled proteins analyzed by 2D-SDS-PAGE. Arrow indicates position of rIGFBP-2.

performed to confirm these observations at the molecular level as well as address potential mechanisms for developmental and cell-specific expression of this protein. As shown in Figure 3-9, astrocytes from 21-day rat brain (lane 2) demonstrate much higher levels of IGFBP-2 mRNA than do neonatal rat brain astrocytes (lane 1). RNA from neonatal rat brain neurons (lane 3) showed very little hybridization to the IGFBP-2 probe. Quantitation of hybridized radioactivity using the Betagen™ radiodensitometric scanner revealed 5-fold higher levels of hybridization signal in astrocytes from 21-day rat brain (65.0 cpm, over background) than from neonatal rat brain (12.7 cpm, over background)

Although very little hybridization was detected visually in lane 3, containing neonatal neuron RNA, quantitation revealed that some signal was present (8.1 cpm, over background)

Regulation of IGFBP-2 in astrocyte cultures by dibutyryl cyclic adenosine monophosphate (diBucAMP)

Regulation of IGFBP-2 secretion by 21-day rat brain astrocytes in response to the differentiating agent diBucAMP was analyzed by Western blotting of conditioned medium from these cells. Following 24 hr treatment of 21-day rat brain astrocytes with increasing concentrations of diBucAMP (1 nM-1 mM), proteins in equal volumes (250 µl) of culture medium from these cells were separated by 1D-SDS-PAGE, transferred to nitrocellulose, and Western blotted with anti-AGSP-

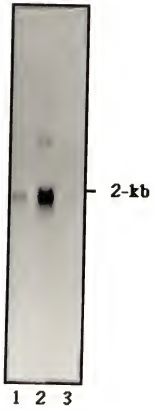

Figure 3-9. Expression of IGFBP-2 mRNA by astrocytes and neurons cultured from rat brain. Astrocytes from neonatal and 21-day rat brain and neurons from neonatal rat brain were maintained in culture under serum-free conditions for 24 hr. Total astrocyte and neuron cellular RNA was extracted, separated on 1.2% agarose gels and blotted. The blot was probed with a 531 bp SmaI-SacI fragment of cDNA encoding rIGFBP-2 as described in *Methods*. *Lane 1*, Neonatal rat brain astrocytes. *Lane 2*, 21-day rat brain astrocytes. *Lane 3*, Neonatal rat brain neurons. Each lane contains 20 µg RNA.

38K/IGFBP-2 antiserum as described in *Methods*. As shown in Figure 3-10, diBucAMP (1 nM-1 mM) produced significant, dose-associated increases in immunoreactive IGFBP-2 secretion by astrocytes from 21-day rat brain, with maximal stimulation observed at 0.5 mM. Nonspecific binding to bands M_r 50-60,000, observed previously was especially prominent in lane 6 of this blot, and cannot be explained. Since equal volumes of protein-containing medium were loaded to each gel, the possibility that more total protein was present in this sample may explain this observation. DiBucAMP-induced IGFBP-2 secretion in astrocytes was similarly detected following both 6 hr exposure to drug (data not shown).

Treatment of 21-day rat brain astrocytes with diBucAMP (0.5-1 mM) also caused marked changes in astrocyte morphology (Figure 3-11). In two experiments, changes in astrocyte morphology were not observed at doses of diBucAMP lower than 0.5 mM. Furthermore, this effect was transient, lasting less than 24 hr, at which time astrocytes returned to a characteristic flat, polygonal morphology.

Discussion

Astrocytes from maturing rat brain preferentially synthesized and secreted a protein, AGSP-35K, identified as the M_r 30-35,000 insulin-like growth factor binding protein, IGFBP-2. Identification of AGSP-35K as IGFBP-2 was based upon biochemical, immunological, and functional criteria as

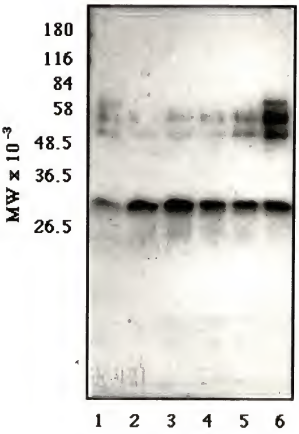

Figure 3-10. DiBucAMP stimulation of IGFBP-2 secretion by astrocytes cultured from 21-day rat brain. Astrocytes were maintained in culture under serum-free conditions for 24 hr in the presence of diBucAMP (1 nM - 1 mM). Conditioned medium was collected and proteins in 250 µl aliquot samples separated by 1D-SDS-PAGE, blotted, and tested with anti-AGSP-35K/IGFBP-2 antiserum. Bound antibody was detected with [^{125}I]-protein A. Medium from untreated astrocytes (lane 1) and astrocytes treated with 1 mM (lane 2), 0.5 mM (lane 3), 1 µM (lane 4), 0.5 µM (lane 5), and 1 nM (lane 6) DiBucAMP are shown.

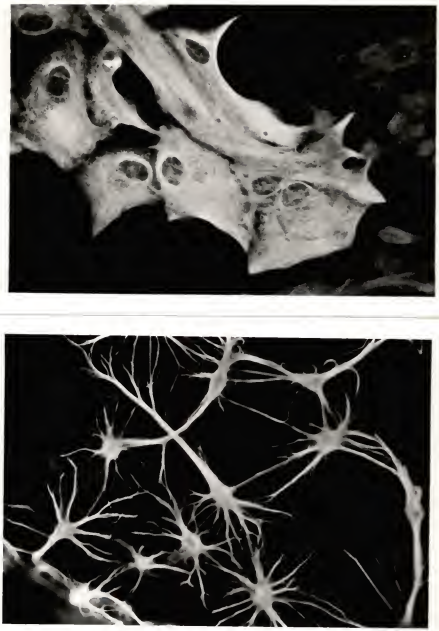

Figure 3-11. DiBucAMP-induced morphologic change in astrocytes cultured from 21-day rat brain. Astrocytes were maintained in culture under serum-free conditions for 6 hr in the presence of 1 mM diBucAMP. Culture medium was removed, cells fixed with 4% paraformaldehyde at 4° C and permeabilized with methanol. Untreated control (*top*) and DiBucAMP-treated (*bottom*) astrocytes were stained with rabbit anti-GFAP antiserum (1:20) and visualized using a rhodamine-conjugated goat anti-rabbit secondary IgG (1:400) as described in *Methods*. Image represents 450X magnification.

well as N-terminal amino acid sequence identity between N-terminal regions of these two proteins. Astrocytes from 14-day, 21-day and adult rat brain secreted high levels of IGFBP-2 whereas small amounts of this binding protein were secreted by astrocytes from neonatal and 7-day rat brain. Neonatal rat brain neurons secreted little of this binding protein.

Northern analysis of total cellular RNA from these cells using an IGFBP-2 cDNA probe were conducted to confirm and extend information concerning developmental regulation of IGFBP-2 in cultured astrocytes. Expression of IGFBP-2 mRNA was significantly higher in 21-day versus neonatal rat brain astrocytes and neurons, suggesting that developmental and cell-specific secretion of IGFBP-2 may be regulated at the transcriptional level.

Regulation of this binding protein in astrocyte cultures of 21-day rat brain astrocytes was also addressed. DiBucAMP, an agent which promotes differentiation and stellation of astrocytes, stimulated secretion of IGFBP-2 from these cells. The relationship between stimulation of IGFBP-2 secretion and differentiative changes induced by diBucAMP in 21-day rat brain astrocytes is at present unclear, but may involve regulation of IGF proliferative action in these cells.

The insulin-like growth factors (IGF-1 and 2) are peptides structurally related to insulin and are thought to play an integral role in the development and differentiation of a number of tissues, including brain (Daughaday and

Rotwein, 1989). The presence of IGF-1 and 2 mRNA and protein in the fetal and early postnatal CNS is well-documented (Lund *et al.*, 1986; Latteman *et al.*, 1989) where these peptides act by autocrine and paracrine mechanisms to influence the growth and differentiation of neurons (Recio-Pinto and Ishii, 1984a; Recio-Pinto *et al.*, 1984b), astrocytes (Han *et al.*, 1987) and oligodendrocytes (McMorris *et al.*, 1986). Unlike peripheral tissues in which IGF-2 protein and mRNA levels decline and IGF-1 predominates postnatally (Daughaday and Rotwein, 1989), brain and cerebrospinal fluid (CSF) maintain relatively high amounts of IGF-2 mRNA (Brown *et al.*, 1986; Murphy *et al.*, 1987) and protein (Hossenlopp *et al.*, 1986) as well as low levels of IGF-1. Although specific role(s) of IGF-1 and 2 in brain physiology are not understood, the persistence of IGF 2 in the developing and mature brain is suggestive of a unique role of this peptide in the CNS.

In body fluids such as CSF, the IGFs circulate bound to specific carrier proteins which themselves demonstrate a complex pattern of developmental regulation (Romanus *et al.*, 1986). At least three related but genetically distinct binding proteins have been described. These include IGFBP-1, an approximately M_r 30,000 binding protein purified from human amniotic fluid (Povoa *et al.*, 1984) and placenta (Bohn and Krause, 1980), human IGFBP-3, the M_r 38-40,000 glycosylated subunit of the M_r 150,000 complex found in adult serum (Martin Baxter, 1986), and rat IGFBP-2, the M_r 30-35,000 non-glycosylated binding protein originally isolated

from rat BRL-3A liver cell line (Mottola *et al.*, 1986; Lyons *et al.*, 1986; Brown *et al.*, 1989). IGFBP-2 has received special attention in recent years for its co-expression with IGF-2 and persistence in the adult central nervous system (Yang *et al.*, 1990; Tseng *et al.*, 1989; Romanus *et al.*, 1989, Margot *et al.*, 1989).

In addition to their role in IGF transport, these binding proteins may serve an important function in the regulation of tissue responses to the IGFs. Purified preparations of the IGF binding proteins have been shown to both potentiate (Elgin *et al.*, 1987) as well as inhibit (Knauer and Smith, 1980; Drop *et al.*, 1979; DeMellow and Baxter, 1988) biological actions of IGFs in several tissues, including brain (Han *et al.*, 1988). The role of IGFBP:IGF interactions in the modulation of IGF activity is still largely undefined; however, an increasing body of evidence suggests that such interactions must be taken into account in analyses of IGF action in the developing brain

To date, several groups have investigated IGF binding protein production by cells from the CNS. Han *et al.*, (1987) first demonstrated that astrocytes cultured from neonatal rats secrete two species of non-glycosylated IGF binding proteins, one identified by immunological and molecular weight criteria as IGFBP-2. Using Western ligand blotting and molecular weight comparisons, Ocrant *et al.*, (1989) reported similar observations concerning IGFBP-2 in primary cultures of neonatal astrocytes and neurons as well as in

B104 neuroblastoma cells. In contrast, Yang *et al.*, (1990) found neither IGFBP-2 mRNA nor immunoreactive IGFBP-2 in B104 neuroblastoma or C6 glioma cells. Tseng *et al.*, (1989) have also demonstrated high levels of IGFBP-2 mRNA in choroid plexus by *in situ* hybridization and did not exclude the possibility that astrocytes or neurons synthesize IGFBP-2 mRNA. N-terminal sequence analysis, Western and Western ligand blot, and Northern blot data presented here conclusively identify IGFBP-2 in cultured 21-day rat brain astrocytes and indicate limited production of IGFBP-2 by neonatal astrocytes. A neuronal source of this binding protein was not detected at significant levels in these studies. Differences in neuronal culture techniques may explain this discrepancy.

It is of particular interest to compare the secretion of IGFBP-2 by astrocytes cultured from neonatal, 7-day, 14-day, 21-day and adult rat brain. Our data indicate a dramatic increase in brain expression of this protein between 7 and 14 days postnatally. Several possibilites may explain this observation. First, the complexity of the developing nervous system and the multiple effects of the IGF^s on different cells of the brain suggest that the brain may demonstrate developmentally-regulated sensitivity to these hormones. Such regulation could be at the level of IGF expression, IGF receptor expression or coupling or IGF binding protein activity. That IGF binding proteins have been shown to both enhance and inhibit IGF action in several cell types may

support a role of these proteins in regulation of IGF action during development. Alternatively, it is possible that our culture technique may select for a particular subset of astrocytes which are present in higher amounts in 14-day, 21-day, and adult brain versus neonatal and 7-day brain. Intersestingly, Miller *et al.*, (1985) have shown that in rat optic nerve,type-1 astrocytes are present at birth, whereas type-2 astrocytes appear at the beginning of the second postnatal week. It is unlikely that cultures from older rats contain a significant percentage of IGFBP-2-producing non-astrocytic cells because: *(i)* 95-98% of cells in neonatal and 21-day rat brain astrocyte cultures are polygonal cells which stain positively for the astrocyte-specific glial fibrillary acidic protein (GFAP) (Olson *et al.*, 1989) *(ii)* two-dimensional SDS-PAGE profiles of cellular and secreted proteins from neonatal and 21-day astrocytes are largely homologous, and *(iii)* astrocytes in culture from neonatal and 21-day rat brain demonstrate similar stellation patterns when treated with 1 mM dibutyryl cyclic AMP (data for neonatal astrocytes not shown).

Demonstration of diBucAMP induction of IGFBP-2 secretion by astrocytes, perhaps associated with differentiation of these cells, raises an intriguing hypothesis for IGF:IGFBP interactions in developing astrocytes. We propose that IGF "drive" for undifferentiated astrocyte cell division (Shemer *et al.*, 1987) is modulated by an autocrine mechanism during differentiation of these cells via increased expression of

IGF-inhibitory IGF binding proteins, including IGFBP-2. Future experiments to address this hypothesis may include determination of: IGFBP-2 mRNA levels in astrocytes treated with diBucAMP; diBucAMP effects on IGF-stimulated DNA synthesis in astrocytes; IGF effects on the astrocyte stellation reaction induced by diBucAMP; anti-AGSP-35K antiserum effects on IGF-induced DNA synthesis; and IGF receptor numbers and activity on diBucAMP-treated cells.

To our knowledge, this is the first demonstration of IGF binding proteins in astrocytes from mature rat brain. These *in vitro* observations correlate well with those of several groups which have identified immunoreactive IGFBP-2 in CSF from both adult rat (Tseng *et al.*, 1989; Lamson *et al.*, 1989) and human (Romanus *et al.*, 1989; Lamson *et al.*, 1989). This culture technique may therefore be useful to delineate factors regulating IGF binding proteins in the developing brain.

CHAPTER 4
CHARACTERIZATION OF ANGIOTENSIN II RECEPTORS AND ANGIOTENSIN II-INDUCED CHANGES IN MACROMOLECULAR SYNTHESIS IN NEONATAL AND 21-DAY RAT BRAIN ASTROCYTES

Introduction

The octapeptide Ang II, classically known as the principle bioactive peptide of the systemic renin-angiotensin system (RAS), exerts a variety of actions on cardiovascular, renal, endocrine and nervous systems. Ang II effects pertinent to regulation of circulatory homeostasis are well-characterized and include pressor responses via stimulation of vascular smooth muscle constriction and conservation of salt and water via stimulation of aldosterone biosynthesis in the adrenal zona glomerulosa. The existence of distinct yet interdependent systemic and tissue RAS is now widely appreciated and the spectrum of Ang II activity has been extended considerably (Mendelsohn, 1985). Ang II is a potent stimulator of cardiac rate and contractility independent of adrenergic input (Bonnardeaux and Regoli, 1974). Ang II also modulates pituitary release of prolactin, growth hormone and ACTH (Steele *et al.*, 1981; Aguilera *et al.*, 1982). Like glucagon, Ang II regulates hepatic glucose metabolism via stimulation of both glycogenolysis and gluconeogenesis (Garrison and Wagner, 1982).

Physiologic effects of Ang II are initiated by binding of this peptide to specific high affinity Ang II receptors present on a wide variety of peripheral tissues including adrenal gland (Goodfriend and Lin, 1970), vascular smooth muscle (Gunther *et al.*, 1980), myocardium (Rogers, 1984), kidney (Mendelsohn *et al.*, 1983) and liver (LaFontaine *et al.*, 1979). In most Ang II-sensitive tissues, Ang II receptors exhibit a single high affinity state with K_d values in the 0.1-5 nanomolar range. Ang II-occupation of its receptor initiates two signal transducing cascades in some cell types: 1) hydrolysis of polyphosphatidyl inositol lipids, leading to protein kinase C activation and calcium mobilization, and 2) inhibition of adenylate cyclase (Garcia-Sainxz, 1987). Recent evidence indicates that these dual responses may be mediated by distinct subtypes of the Ang II receptor (Whitebread *et al.*, 1989; Chiu *et al.*, 1989). Although the Ang II receptor has been well-characterized pharmacologically, biochemical analysis of this receptor is still at an early stage. Covalent cross-linking and photoaffinity labelling studies indicate that the Ang II receptor is composed of multiple subunits, each approximately 68,000 M_r (Paglin *et al.*, 1982; Capponi *et al.*, 1980). The number of subunits in the native receptor and the sequence and structure of these subunits are presently unknown.

In recent years the presence of a distinct RAS in brain has been established and has been implicated in central control of blood pressure. This view is based upon

observations that centrally injected Ang II causes profound increases in blood pressure in normotensive rats, that all components of the renin-angiotensin system are found in the brain (Ganong, 1984, Phillips, 1987, Severs and Daniels-Severs, 1973), and that increased levels of Ang II and Ang II receptors are seen in brains of spontaneously hypertensive rats (Phillips and Kimura, 1988; Maclean *et al.*, 1990). Cardiovascular effects of Ang II have been attributed to the modulation of sympathetic activity of neurons in relevant cardioregulatory centers of the brain (Ganong, 1984, Phillips, 1987, Sumners *et al.*, 1983a). *In vitro* studies with neurons in primary culture support this hypothesis: Ang II modulates both catecholamine synthesis and reuptake by these cells (Maclean *et al.*, 1990, Sumners *et al.*, 1986b), and elevated levels of receptors for this peptide have been found in neurons cultured from spontaneously hypertensive rats (Raizada *et al.*, 1984).

Central effects of Ang II are mediated by specific high affinity receptors for Ang II which have been demonstrated and localized in the brain (Mann, 1982; Mendelsohn *et al.*, 1984). These receptors have been found in several brain areas including septum, midbrain, thalamus, hypothalamus, and medulla oblongata. Autoradiographic localization indicates high Ang II receptor densities in areas where Ang II is known to exert behavioral, endocrine and physiological actions the brain, including the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO), areas

involved in central cardiovascular control (Landas *et al.*, 1980; Mendelsohn *et al.*, 1984; Simpson, 1981). Furthermore, immunohistochemical staining of Ang II demonstrates good correlation between peptide and receptor distributions (Healy and Printz, 1984a;Healy and Printz, 1984b).

Receptors for Ang II have been demonstrated in primary cultures of neurons (Raizada *et al.*, 1984). In these cultures, Ang II receptors modulate catecholamine metabolism and concentration (Sumners *et al.*, 1986b; Sumners *et al.*, 1983b). These receptors are regulated by catecholamines acting via alpha$_1$-adrenergic receptors (Sumners and Raizada, 1984; Sumners and Raizada, 1986a), by protein kinase C agonists (Sumners *et al.*, 1987a) and by mineralocorticoids (Wilson *et al.*, 1986). These observations correlate well with the hypothesis that central actions of Ang II are the result of modulation of neuronal and neurosecretory activity in specific brain regions (Unger *et al.*, 1988).

In addition to a neuromodulatory role of Ang II, evidence now exists for a distinct Ang II system in CNS astrocytes. Astrocytes in culture contain both angiotensinogen mRNA (Kumar *et al.*, 1988; Intebi *et al.*, 1990) and Ang II (Hermann *et al.*, 1988a;Hermann *et al.*,1988b). Furthermore, these cells possess immunoreactive Ang II and angiotensinogen mRNA *in vivo* (Deschepper *et al.*, 1986; Stornetta *et al.*, 1988). Distinct Ang II receptors have also been demonstrated on CNS astrocytes. Specific [^{125}I]-Ang II binding has been described in cultures of

neonatal rat brain astrocytes (Raizada *et al.*, 1987) and has been confirmed in these cells and in astrocytes from spinal cord using light microscopic autoradiography (Raizada *et al.*, 1987; Simmonet *et al.*, 1988). Although the presence of an Ang II system in astrocytes is well documented, little is currently understood of its role in normal brain physiology.

In addition to its blood pressure and volume-regulatory effects, evidence now suggests that Ang II may regulate both cellular growth and differentiation in a number of tissues. Specific binding sites for the Ang II receptor antagonist, [^{125}I]-sar^{1}, ile^{8}-Ang II have been demonstrated in several fetal tissues including the adrenals, kidney, liver and smooth muscle of blood vessels of the embryologic day 19 (E19) rat fetus (Millan *et al.*, 1989). Ang II induces increases in DNA synthesis, protein synthesis, and cell size in cultured vascular smooth muscle cells (Campbell-Boswell and Robertson, 1981; Geisterfer *et al.*, 1988; Berk *et al.*, 1989). Ang II stimulates expression of the growth associated proto-oncogenes c-fos and c-myc (Naftilan *et al.*, 1989b), and induces angiogenesis (Fernandez *et al.*, 1985). Recent identification of the Ang II receptor as the product of the *mas* oncogene provides further evidence for a role of Ang II in growth regulation (Jackson *et al.*, 1988). These observations suggest that Ang II may play an integral role in growth and development of certain cell populations, in addition to its classical role in circulatory homeostasis.

Observations of distinct Ang II receptors on CNS astrocytes, coupled with new evidence for a growth promoting role of Ang II, have raised the question whether Ang II may demonstrate unique roles in brain pertinent to growth and development this cell type in the CNS. To address this issue, astrocytes from neonatal and 21-day rat brain were established in primary culture and examined for the presence of Ang II receptors and Ang II-mediated growth effects. Specifically, these studies characterized [^{125}I]-Ang II binding in neonatal and 21-day rat brain astrocytes and assessed Ang II-stimulated incorporation of [^{3}H]-thymidine into DNA and [^{35}S]-methionine into proteins in these two cell populations.

Results

Distinct binding sites for [^{125}I]-Ang II were demonstrated in astrocyte cultures prepared from neonatal and 21-day rat brain. Specific binding of [^{125}I]-Ang II to astrocytes in both cultures increased with time and reached a plateau between 3 and 5 hours at 4° C (Figure 4-1). At four hours, neonatal and 21-day rat brain astrocytes bound 2.0 and 12.0 fmol [^{125}I]-Ang II/mg protein, respectively. Nonspecific binding in both cultures did not exceed 5-7% during this period. Acid dissociation of bound [^{125}I]-Ang II following a 4 hr incubation at 4° C indicated that 94 and 98% of cell-associated [^{125}I]-Ang II was bound to surface sites on

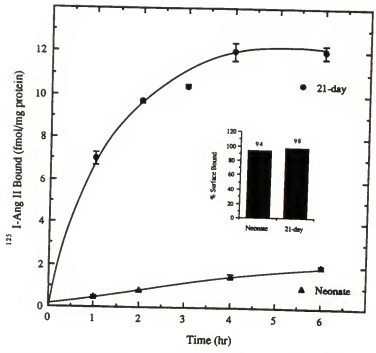

Figure 4-1. Time dependence of [^{125}I]-Ang II binding to astrocytes cultured from neonatal and 21-day rat brain. Confluent neonatal (▲) and 21-day (●) rat brain astrocyte cultureswere incubated with 0.15 nM [^{125}I]-Ang II (100,000 cpm) in the absence and presence of 1 μM unlabeled Ang II for indicated times at 4° C, and specific binding determined as described in *Methods*. Each time point represents mean ± S.E.M. of three cultures and reflects specific [^{125}I]Ang II binding (fmol/mg protein). *Inset,* Acid dissociation of specifically bound [^{125}I]-Ang II following incubation for 4 hr at 4° C.

neonatal and 21-day rat brain astrocytes, respectively (Figure 4-1, inset).

Specific binding of [^{125}I]-Ang II to both neonatal and 21-day rat brain astrocytes was saturable as a function of Ang II concentration (Figure 4-2). Scatchard analyses of saturation data (Figure 4-3) revealed straight lines indicating a single population of binding sites with a dissociation constant (K_d) of 0.5 nM for both astrocyte cultures. Neonatal and 21-day rat brain astrocytes demonstrated B_{max} values of 25 and 80 fmol/mg protein, respectively.

Specificity of [^{125}I]-Ang II binding in 21-day rat brain astrocytes.was demonstrated by competition analyis of binding sites using unlabeled Ang I, Ang II, Ang III, and sar^1, ile^8-Ang II (Figure 4-4). Unlabeled Ang II inhibited binding of [^{125}I]-Ang II in a dose-dependent manner with an IC_{50} of 0.1 nM. Ang III and Ang I were much less potent in competing for [^{125}I]-Ang II binding sites in these cells, demonstrating IC_{50} values of 0.8 μM and 1.0 μM, respectively. The Ang II receptor antagonist, sar^1, ile^8-Ang II, also inhibited [^{125}I]-Ang II binding in a dose-dependent manner with an IC_{50} of 0.16 nM. This order of potency (Ang II > sar^1, ile^8-Ang II >> Ang III > Ang I) correlates well with that reported for neonatal rat brain astrocytes (Raizada *et al.*, 1987).

Neonatal and 21-day rat brain astrocytes in culture were used to assess potential Ang II-stimulation of astrocyte DNA and protein synthesis in developing brain. Comparison of

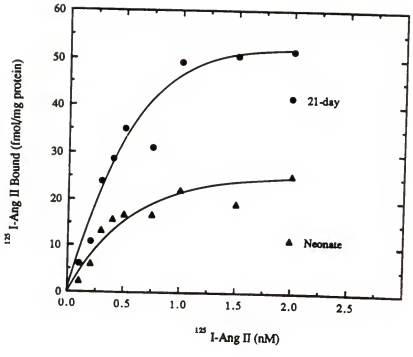

Figure 4-2. Concentration dependence of [^{125}I]-Ang II binding to astrocytes cultured from neonatal and 21-day rat brain. Confluent neonatal (▲) and 21-day (●) rat brain astrocyte cultures were incubated with indicated concentrations of [^{125}I]-Ang II in the absence and presence of 1 μM unlabeled Ang II for 4 hr at 4° C, and specific binding determined as described in *Methods*. Each point represents mean ± S.E.M. of three cultures and reflects specific [^{125}I]-Ang II binding (fmol/mg protein).

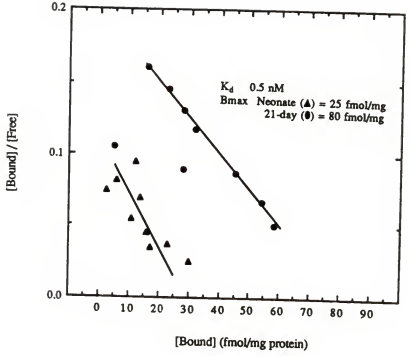

Figure 4-3. Scatchard analysis of [^{125}I]-Ang II binding to astrocytes cultured from neonatal and 21-day rat brain. Saturation data for [^{125}I]-Ang II binding to neonatal (▲) and 21-day (●) astrocytes in culture were analyzed by the method of Scatchard. The data are plotted as the ratio of the amount of specifically bound ligand to free ligand versus amount of specifically bound ligand (fmol/mg protein). Each point represents the mean of three cultures.

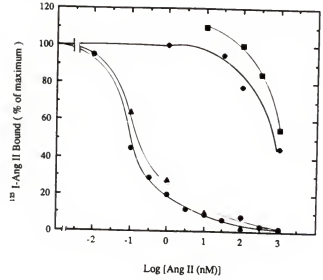

Figure 4-4. Competition analysis of [^{125}I]-Ang II binding to astrocytes cultured from 21-day rat brain. Confluent 21-day rat brain astrocyte cultures were incubated with 0.15 nM [^{125}I]Ang II (100,000 cpm) in the presence of indicated concentrations of unlabeled Ang I (■), Ang II (●), Ang III (♦) or sar^{1}, ile^{8}-Ang II (▲) for 4 hr at 4° C, and specific binding determined as described in *Methods*. Each point represents the mean of three cultures and reflects % of maximum [^{125}I]-Ang II binding in the presence of inhibitors.

[^{3}H]-thymidine incorporation into TCA precipitable DNA in neonatal and 21-day rat brain astrocytes under serum-free conditions indicate no significant differences in basal DNA synthesis between these cells (Figure 4-5). Cells in both cultures were also equally sensitive to stimulation of [^{3}H]-thymidine incorporation into DNA after 24 hr pretreatment with 10% fetal bovine serum. In response to 24 hr pretreatment with 1 nM Ang II, astrocytes from 21-day rat brain demonstrated marked increases (2-4 fold, 5 experiments) in DNA synthesis, whereas astrocytes from neonatal rat brain demonstrated modest (< 2 fold, 3 experiments), but statistically significant ($p < 0.001$), increases in DNA synthesis in response to Ang II.

Ang II induction of [^{3}H]-thymidine incorporation into DNA of 21-day rat brain astrocytes was also dose-dependent (Figure 4-6). The EC_{50} for Ang II-stimulated DNA synthesis in these cells following 24 hr pre-treatment was 0.5 nM and maximal stimulation of incorporation (3-fold over basal) was observed at concentrations of 1 nM Ang II and higher. Frequently, 24 hr treatment of cells with Ang II concentrations 10 μM and higher resulted in lower [^{3}H]-thymidine incorporation than that observed for Ang II concentrations in the nanomolar range.

Ang II stimulation of [^{3}H]-thymidine incorporation into DNA in 21-day rat brain astrocytes was blocked by co-administration of the Ang II receptor antagonist sar^1, ile^8-Ang II. Stimulation of DNA synthesis following 24 hr

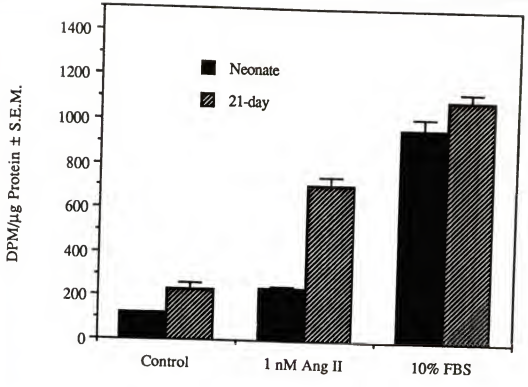

Figure 4-5. Angiotensin II stimulation of [^{3}H]-thymidine incorporation into neonatal and 21-day rat brain astrocytes. Subconfluent cultures of neonatal (*stipled bars*) and 21-day (*solid bars*) rat brain astrocytes were incubated in the presence of 1 nM Ang II or 10% FBS for 24 hr and assayed for incorporation of [^{3}H]-thymidine as described in *Methods*. Data are shown as the mean ± S.E.M. of three cultures and reflects DPM [^{3}H]-thymidine incorporated/µg protein.

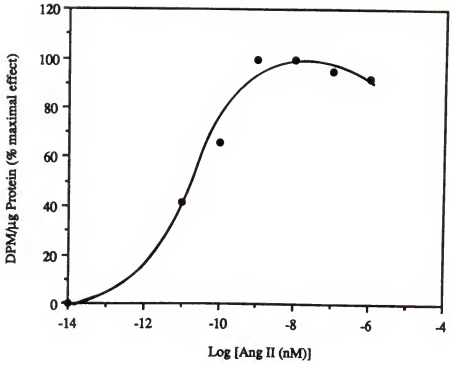

Figure 4-6. Dose-response of induction of [^{3}H]-thymidine incorporation by Ang II in 21-day rat brain astrocytes. Subconfluent cultures of 21-day rat brain astrocytes (•) were incubated with indicated concentrations of Ang II for 24 hr and assayed for incorporation of [^{3}H]thymidine as described in *Methods*. Each point represents the mean of three cultures and reflects % maximal incorporation of [^{3}H]-thymidine (713 ± 30 DPM/µg protein in this experiment).

pretreatment with 10 nM Ang II was partially blocked by co-incubation with 1 μM sar^1, ile^8-Ang II and was no different from control levels in the presence of 10 μM sar^1, ile^8-Ang II (Figure 4-7). Sar^1, ile^8-Ang II alone demonstrated no stimulatory effect at these concentrations (data not shown).

Ang II effects on total protein synthesis was also investigated in cultures of neonatal and 21-day astrocytes. In these experiments, *de novo* synthesis of cellular and secreted proteins was determined by incorporation of [^{35}S]methionine into nondialyzable protein in cell lysate and medium samples from these cells. Basal incorporation of [^{35}S]methionine into cell lysates and secreted proteins during an 8 hr labeling period was comparable in cultures of neonate and 21-day rat brain astrocytes in most experiments. However, as shown in Figure 4-8, 24 hr incubation of 21-day rat brain astrocytes with 1 μM Ang II resulted in 165% and 60% increases in [^{35}S]-methionine incorporation into both cellular and secretory proteins during the 8 hr labeling period, respectively, whereas no significant stimulation of either cellular or secretory protein synthesis was observed at 1 μM (or higher concentrations) Ang II in neonatal rat brain astrocytes.

In separate experiments, maximal induction of [^{35}S]-methionine incorporation into total secretory proteins during 24 hr incubation with 1 μM Ang II was reduced 34-75% by when coincubated with ten-fold higher concentrations of sar^1, ile^8-Ang II (10 μM). This was an inconsistent result since at

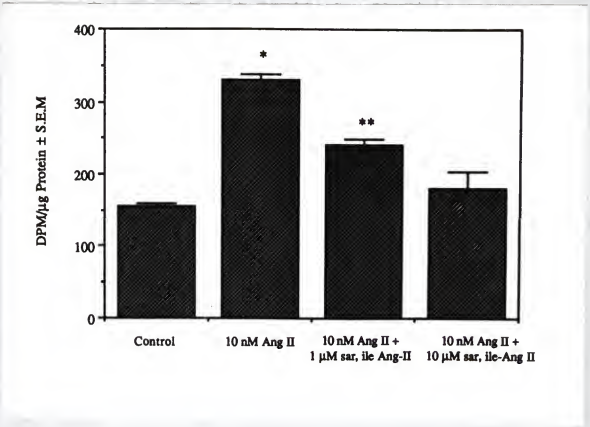

Figure 4-7. Inhibition of Ang II-induced [^{3}H]-thymidine incorporation in 21-day rat brain astrocytes by sar^1, ile^8-Ang II. Subconfluent cultures of 21-day rat brain astrocytes were incubated with 10 nM Ang II in the absence and presence of indicated concentrations of sar^1, ile^8-Ang II for 24 hr and assayed for incorporation of [^{3}H]-thymidine as described in *Methods*. Each column represents the mean ± S.E.M. of three cultures and reflects DPM [^{3}H]-thymidine incorporated/µg protein. *Asterisk*, $p < 0.001$ from control. *Double asterisk*, $p < 0.001$ from Ang II-treated cultures.

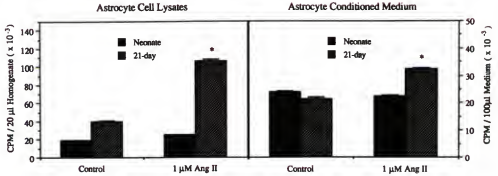

Figure 4-8. Angiotensin II stimulation of [^{35}S]-methionine incorporation into cellular and secretory proteins from neonatal and 21-day rat brain astrocytes. Confluent cultures of neonatal (solid bars) and 21-day (stipled bars) rat brain astrocytes were incubated with 1 µM Ang II under serum-free conditions for a total of 24 hr, the last 8 hr in the presence of 100 µCi/dish [^{35}S]-methionine. Cell lysate and conditioned medium samples were collected, processed, and analyzed as described in *Methods*. Each bar represents the mean ± S.E.M. of triplcate measurements from a single representative culture and reflects CPM [^{35}S]-methionine incorporated/100 µl (medium) or 20 µl (cell lysates).

concentrations 10 μM and higher, potentiation of 1 μM Ang II-induced [^{35}S]-methionine incorporation by sar^{1}, ile^{8}-Ang II was often seen.

Discussion

Astrocytes from neonatal and 21-day rat brain demonstrate specific high affinity receptors for Ang II. Receptors on these cells were indistinguishable with respect to dissociation constants (0.5 nM each) and were similar in number (25 and 80 fmol/mg protein for neonatal and 21-day, respectively). Competition analysis of [^{125}I]-Ang II binding to 21-day rat brain astrocytes revealed a potency profile (Ang II > sar^{1}, ile^{8}-Ang II >> Ang III > Ang I) identical to that reported for neonatal rat brain astrocytes (Raizada *et al.*, 1987). These *in vitro* data suggest that Ang II receptors on astrocytes are pharmacologically similar during this period of postnatal development.

Distinct high affinity Ang II receptors have been demonstrated in relevant hypothalamic and brainstem cardioregulatory centers by several investigators (reviewed in Mendelsohn, 1985). However, such Ang II receptor analyses using membrane preparations of specific brain areas and autoradiographic studies have provided few definitive answers with regard to the cellular distribution of Ang II receptors or Ang II-mediated cellular events. Recently, the ability to grow purified populations of astrocytes and neurons in

culture has allowed for characterization of Ang II receptors at the cellular level.

The pharmacologic characteristics, regulation and biochemical considerations of Ang II receptors present on neonatal hypothalamic and brainstem neurons and glia in culture have been recently reviewed (Sumners *et al.*, 1990). Reported K_d and B_{max} values for Ang II receptors in neonatal neuronal cultures range from 0.69-1.13 nM and 94-163 fmol/mg protein, respectively, and in neonatal astrocyte cultures are 1.1nM and 110 fmol/mg protein, respectively. Competition binding studies in intact cells have revealed distinct potency series for Ang II receptors on these cells. The competition profile of neurons from spontaneously hypertensive (SHR) rat brain (Ang II >> saralasin > Ang III > Ang I) Raizada *et al.*, 1984) is distinguishable from that of intact hypothalamic and brainstem astrocytes and that typically observed in brain membranes (sarlasin > Ang II > sar,ile-Ang II >> Ang III > Ang I). However, Ang II receptor competition analyses of membranes prepared from neurons and astrocytes of hypothalamus and brainstem revealed comparable Ang II competition profiles (Sumner *et al.*, 1990). From these data it has been concluded that both neonatal rat brain neurons and astrocytes possess specific high affinity Ang II receptors which are pharmacologicaly similar to those present in brain membranes.

The K_d and B_{max} values reported here for neonatal and 21-day whole brain astrocytes are slightly different from, but

in general agreement with, previously reported values for rat brain astrocyte cultures (Raizada *et al.*, 1987). This comparison suggests that astrocytes cultured from whole brain demonstrate similar pharmacologic properties to those cultured from brain stem and hypothalamus. This observation is in contrast to the current view that few physiologically relevant Ang II receptors are present in regions other than brainstem and hypothalamus (Mendelsohn, 1985). However, in support of these observations, dissociation constants from this study are in good agreement with those reported for membrane-rich fractions of whole rat brain (Bennett and Snyder, 1976).

To our knowledge these are the first studies addressing potential developmental changes in brain Ang II receptors using an *in vitro* model. Ang II receptor levels have been shown to be developmentally regulated in rat brain membrane and synaptosomal preparations (Baxter *et al.*, 1980; Myers *et al.*, 1989). These studies indicate that maximal [^{125}I]-Ang II binding to membranes and synaptosomes from various brain regions (10-15 fmol/mg protein) occurs 2-28 days postnatally, and are as much as 10 times higher than in neonatal (< 1-day) and adult (> 63 days) rats. In principle, results from this study agree with these earlier observations. Less than three-fold differences in B_{max} values and no differences in K_d values were found between astrocytes cultured from neonatal versus 21-day rat brain. [^{125}I]-Ang II binding competition potency profiles for 21-day rat brain astrocytes

described here and for neonatal astrocytes previously reported were also similar (Raizada *et al.*, 1987). Furthermore, maximum Ang II binding (50 fmol/mg protein) determined in these studies was higher than reported with either membranes (10 fmol/mg protein) (Baxter *et al.*, 1980) or synaptosomes (12-15 fmol/mg protein) (Myers *et al.*, 1989). Discrepancies between current and previous observations may reflect differences in: (i) Ang II receptor behaviour in intact astrocytes versus whole brain-derived membrane and neuronal synaptosome preparations; (ii) loss of developmental integrity in this receptor system under *in vitro* conditions; or (iii) changes in apparent binding due to uptake of [^{125}I]-Ang II into intact astrocytes. Although we cannot exclude the first possibility, the second and third possibilities are unlikely. Substantial evidence indicates that rat brain astrocytes cultured from animals of different ages maintain distinct propeties including protein secretion (Olson *et al.*, 1989; Olson *et al.*, 1990; Schurch-Rathgeb and Monard, 1985), muscarinic acetylcholine receptor expression (Ashkenazi *et al.*, 1989), and adrenergic receptor expression (Raizada, in preparation). However, whether these differences are true "developmental" differences or may reflect selection of distinct populations of astrocytes is unresolved at the present time. Finally, differences in observed binding of [^{125}I]-Ang II due to receptor-ligand internalization in intact cells would be minimal since in these experiments, conducted

at 4° C, internalized [^{125}I]-Ang II binding was less than 5% of total.

In contrast to [^{125}I]-Ang II binding, significant differences were observed in responses of these two astrocyte populations when treated with Ang II. Astrocytes from 21-day rat brain demonstrated significant, dose-dependent increases in DNA as well as cellular and secretory protein synthesis in response to Ang II. These responses were significantly attenuated in the presence of sar^{1}, ile^{8}-Ang II indicating that these effects are indeed Ang II receptor mediated. Astrocytes from neonatal rat brain, on the other hand, failed to respond to Ang II in this manner, even at doses 100 fold higher than those inducing effects in 21-day rat brain astrocytes.

As outlined in the introduction, a growing body of evidence suggests that Ang II may act to control growth and differentiation of certain cell populations. Cells of vascular smooth muscle have been most thoroughly investigated in this regard based upon the hypothesis that an oversensitvity to Ang II cause the marked hypertrophy of these cells often seen in hypertension (Liu *et al.*, 1988). Indeed, *in vitro* data have supported this hypothesis: Ang II increases the growth rate of subconfluent human aortic smooth muscle cells (Campbell-Boswell and Robertson, 1981); Ang II increases cell size, DNA and protein content in smooth muscle cells without increaseing cell number (Geisterfer *et al.*, 1988); and Ang II induces several growth associated events

such as c-fos and c-myc expression, and PDGF A-chain secretion in cultured smooth muscle cells (Naftilin *et al.*, 1989a; Naftilan *et al.*, 1989b).

Evidence from the present study indicate that growth promoting actions of Ang II extend to astrocytes of rat brain. Our observations further indicate that astrocytes from 21-day animals are sensitive to Ang II, whereas those from neonatal animals are not. Several possibilities may explain these differences. The slight increase in the B_{max} value for 21-day versus neonatal rat brain astrocytes alone is not, in our estimation, adequate to explain these differential responses at these concentrations of Ang II; however, these astrocytes may express subtypes of Ang II receptors differentially coupled to their effector systems. Differential expression of muscarinic acetylcholine receptor subtypes in fetal versus postnatal rat brain mediates developmentally-regulated proliferative responses of these cells to carbachol (Ashkenazi *et al.*, 1989). Ang II sensitivity in these cells may be similarly regulated. Recent demonstration of subtypes of Ang II receptors may be relevent to this hypothesis (Whitebread *et al.*, 1989; Chiu *et al.*, 1989). Alternatively, our culture technique may select for a subset of Ang II-sensitive astrocytes present in greater proportions in 21-day than in the neonatal rat brain. Interestingly, type II astrocytes in the rat CNS demonstrate a burst of proliferation 14 days postnatally and their presence in the 21-day rat brain cultures could possibly

contribute to these observations. However, this is unlikely since > 95% of the cells in both neonatal and 21-day cultures demonstrate a flat, polygonal morphology characteristic of type I astrocytes. Nevertheless, these or other subpopulations of type I astrocytes could express Ang II receptors linked to growth regulatory pathways similar to those of vascular smooth muscle cells described previously. Finally, Ang II stimulation of 21-day rat brain astrocytes may induce the synthesis and secretion of other peptides which could act in an autocrine manner to effect a proliferative response in these cells. Observations that Ang II induces PDGF A chain (Naftilin *et al.*, 1989a) secretion provides support for this hypothesis. Observed differences in Ang II responses between neonatal and 21-day rat brain astrocytes may then be due to differences in Ang II-induced secretion of such factors, sensitivity to these factors, or both.

That Ang II caused a dose-dependent and antagonist-inhibitable increase in DNA and protein synthesis in astrocytes from 21-day but not neonatal animals raises exciting possibilities as to the potential role of this peptide in control of astrocyte populations in the mature brain. Preliminary observations from this study, not included in this dissertation, that Ang II induces c-fos expression from 21-day, but not neonatal rat brain astrocytes, underscore the possibility of differential growth responses of these cells to Ang II during development. These

results may seem anomalous since proliferation of astrocytes occurs during development of the CNS but is rarely thought to occur under normal physiological conditions in the mature brain. It is well known, however, that astrocytes undergo marked proliferation (gliosis) and swelling (astrocytosis) following brain trauma and in several disease processes including multiple sclerosis (Billingsley *et al.*, 1982) and hypertension (Magnus *et al.*, 1977). Moreover, these processes may retard the processes of CNS repair and axonal regrowth in these situations. The possibility that cultured 21-day rat brain astrocytes are "reactive astrocytes", subject to hormonal control of proliferation is particularly exciting since type-1 astrocytes,cells in greatest abundance in these cultures, resemble reactive astrocytes which proliferate in adult brain in response to injury (Miller *et al.*, 1986). If indeed Ang II plays a pivotal role in such developmentally-staged reactive processes, then therapeutic strategies for its control may well revolve around currently available drugs such as Ang II receptor antagonists and converting enzyme inhibitors. Furthermore, if astrocyte-derived gliomas or gliobastomas respond to Ang II like astrocytes in primary culture, then similar pharmacologic approaches may be of benefit in the treatment of these diseases as well. At present, however, these possibilities are speculative and require further investigation, especially conclusive evidence of Ang II-induced increases in astrocyte cell number.

In summary, results from binding studies reported here reveal that neonatal and 21-day rat brain astrocytes demonstrate pharmacologically similar Ang II receptors, while Ang II receptor-mediated responses of these two astrocyte populations to Ang II are widely different. Comparison of these results to previous Ang II receptor data from our laboratory and data from other laboratories obtained using membrane binding and quantitative autoradiography indicate that this culture system is a good model for the study of Ang II receptors and their regulation in developing brain.

This study also indicates that astrocytes from rat brain may be targets for growth promoting actions of Ang II, and that this action may be developmentally restricted to astrocytes from more mature brain. These results have exciting implications for the role of Ang II in control of astrocyte populations during the aging process as well as in several pathalogical processes in the CNS

CHAPTER 5
CHARACTERIZATION AND IDENTIFICATION OF ANGIOTENSIN II-INDUCED SECRETORY PROTEINS IN ASTROCYTES FROM 21-DAY RAT BRAIN

Introduction

Considerable evidence now supports the presence of a distinct renin-angiotensin system (RAS) in the brain having all necessary components including angiotensinogen, renin, Ang I, converting enzyme, Ang II and Ang III produced locally in the CNS. Specific Ang II receptors have been demonstrated on both neurons and astrocytes and are present in regions of the brain coincident with immunohistochemical localization of Ang II. These observations, coupled with those describing profound pressor and dipsogenic effects following direct administration of Ang II to relevant cardioregulatory centers of the brain, have implicated the brain RAS in central control of blood pressure.

Effects of Ang II on brain have always been attributed to its actions on CNS neurons. Microiontophoretic application of Ang II to several brain areas, including the subfornical organ (SFO), preoptic area, and the organum vasculosum of the lamina terminalis (OVLT) produces excitatory neuronal effects (Unger *et al.*, 1985). Ang II also acts directly upon supraoptic neurons to stimulate hypophyseal release of vasopressin and oxytocin (Nicoll and

Barker, 1971). Early *in vitro* studies also suggested that Ang II modulates neuronal release, uptake and metabolism of catecholamines (Severs and Daniels-Severs, 1973). Studies of neurons in primary culture later demonstrated that Ang II regulates catecholamine re-uptake and metabolism by alterations in transporter number and monoamine oxidase activity (Sumners *et al.*, 1990). Thus, both *in vivo* and *in vitro* observations indicate a neuromodulatory role of Ang II in the brain.

In addition to an accepted neuromodulatory role for Ang II in the brain, evidence now suggests that this peptide may have unique effects on CNS astrocytes. Distinct receptors for Ang II (Raizada *et al.*, 1987), angiotensinogen mRNA (Kumar *et al.*, 1988; Intebi *et al.*, 1990) and Ang II (Hermann *et al.*, 1988a; Hermann *et al.*, 1988b) have all been demonstrated in purified populations of astrocytes in culture, while immunoreactive Ang II and angiotensinogen mRNA have been observed in astrocytes *in vivo* (Deschepper *et al.*, 1986; Stornetta *et al.*, 1988).

Investigation into Ang II-coupled second messenger systems and Ang II-mediated catecholamine metabolism in astrocytes and neurons have also revealed striking differences. In neurons, Ang II receptors are negatively coupled to cGMP production, while astrocytic Ang II receptors are coupled to phosphatidyl inositol hydrolysis. In neurons, Ang II elicits changes in catecholamine re-uptake and metabolism by monoamine oxidase, an effect not observed in

astrocytes. Although astrocytes demonstrate "functional" Ang II receptors, nothing is currently known of Ang II action on these cells. As described in Chapter 4, Ang II may behave as a growth factor in these cells, stimulating DNA and protein synthesis. However, biochemical mechanisms involved in this or other astrocyte responses to angiotensin peptides in brain are not understood.

A growing body of evidence now suggests that intercellular communication between astrocytes and other cells of the brain is mediated, at least in part, by proteins secreted by astrocytes. Astrocytes secrete several neurotrophic peptides including nerve growth factor (NGF) (Westermann *et al.*, 1988) and are thought to to be the source of several other neurotrophic and/or growth regulatory peptides including the insulin-like growth factors (IGFs) (Ishii *et al.*, 1990), fibroblast growth factors (FGFs) (Wallicke, 1988), brain-derived growth factor (BDNF) (Barde *et al.*, 1982) and glial-derived protease nexin-1 (GdPN-1) (Geunther *et al.*, 1985). Furthermore, actions of these neurotrophic or other centrally-active factors may be indirect, mediated by release of proteins by astrocytes. Indeed, neurotrophic properties of vasoactive intestinal peptide (VIP) and EGF have been attributed to the production of soluble factors by astrocytes (Wang *et al.*, 1989; Morrison *et al.*, 1987; Brenneman *et al.*, 1987).

A growing appreciation of the importance of astrocyte secreted proteins in development and maintenance of several

CNS cell types coupled with our observations of Ang II action on 21-day rat brain astrocytes has led us to postulate that Ang II may exert biochemical and physiological effects on developing brain via mechanisms involving synthesis and secretion of proteins by astrocytes. To test this hypothesis, astrocyte cultures from neonatal, 7-day, 14-day, 21-day, and adult rat brain were tested for Ang II-induced changes in *de novo* synthesized proteins secreted by these cells using 2D-SDS-PAGE and fluorography.

Results

Characterization of Ang II -induced secretory proteins from neonatal and 21-day rat brain astrocytes.

Effects of Ang II on cultured neonatal and 21-day rat brain astrocyte secretory proteins were determined by 2D-SDS-PAGE analysis of [^{35}S]-methionine-labeled proteins in serum-free conditioned medium from these cells. Representative fluorograms from control and Ang II-treated 21-day, and Ang II-treated neonatal rat brain astrocyte cultures are shown in Figure 5-1. Comparison of profiles of [^{35}S]-methionine-labeled proteins synthesized and secreted from untreated neonatal and 21-day rat brain astrocytes during the eight hour labeling period again indicates that these cells are very similar in their constitutive production of secretory proteins (refer to Figures 3-2, 5-5A). Treatment of astrocytes from 21-day rat brain with 1 μM Ang II for 24

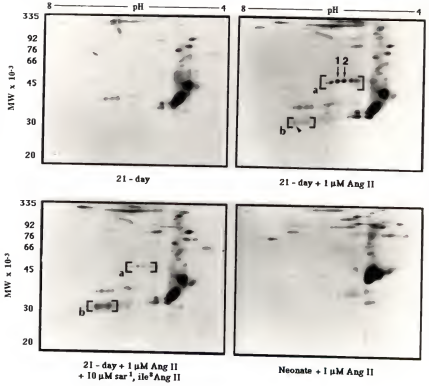

Figure 5-1. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled proteins secreted by astrocytes cultured from neonatal and 21-day rat brain. Astrocytes were extensively rinsed in serum-free MEM containing 10 μM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C without additions or in the presence of 1 μM Ang II. During the final 8 hr of the incubation period, cells were pulsed with 100μCi/dish L-[^{35}S]-methionine. Cultures were terminated and conditioned medium samples processed and analysed by two-dimensional SDS-PAGE followed by fluorography as described in *Methods*. *Top, left*, Total [^{35}S]-methionine-labeled proteins (100,000 cpm) secreted by cultures of 21-day rat brain astrocytes. *Top, right*, [^{35}S]-methionine-labeled proteins secreted by 21-day rat brain astrocytes in the presence of 1 μM Ang II. *Bottom, left*, [^{35}S]-methionine-labeled proteins secreted by 21-day rat brain astrocytes in the presence of 1 μM Ang II and 10 μM sar^{1}, ile^{8} Ang II. *Bottom, right*, [^{35}S]-methionine-labeled proteins secreted by neonatal rat brain astrocytes in the presence of 1 μM Ang II (identical to untreated neonatal cultures). *Brackets*: *a*, Ang II-induced secretory proteins, M_r 55,000 (AISP-55K); *b*, Ang II-induced secretory proteins, M_r 30,000 (AISP-30K). *Arrows*: AISP-55K isoforms sequenced. *Arrowhead*: AISP-30K spot sequenced.

hr induced synthesis and secretion of two groups of proteins: a) M_r 55,000, pI 5.0-5.5 (AISP-55K), and b) M_r 30,000, pI 6.3-7.0 (AISP-30K). Induction of AISP-55K synthesis and secretion by 1 μM Ang II was significantly attenuated in the presence of 10 μM of sar^1, ile^8- Ang II. However, Ang II-stimulated AISP-30K secretion was not significantly inhibited by sar^1, ile^8-Ang II, and in some experiments, concentrations of sar^1, ile^8-Ang II 10 μM and higher, potentiated Ang II induction of AISP-30K. Neonatal astrocytes did not secrete either AISP-55K or AISP-30K in response to any concentrations of Ang II tested (10 nM-10μM), despite the presence of similar Ang II receptors in these cells (Raizada *et al.*, 1987).

Ang II-induced synthesis and secretion of AISP-55K and AISP-30K in 21-day rat brain astrocytes was time dependent (Figure 5-2). Quantitation of these labeled proteins by laser densitometry of 2D-fluorograms indicates significantly elevated levels of labeled AISP-30K and AISP-55K in culture medium as early as two and four hours after addition of 1μM Ang II, respectively.

Fluorograms shown in Figures 5-3 A and B demonstrate that Ang II induction of AISP-30K and AISP-55K was dose-dependent. Densitometry analysis of AISP-30K and AISP-55K spots shown within brackets on fluorograms in Figure 5-3 A and B was performed as described in *Methods*. Graphic representation of densitometric data in Figure 5-4 indicated EC_{50} values of 1.5 nM for Ang II-induced synthesis and

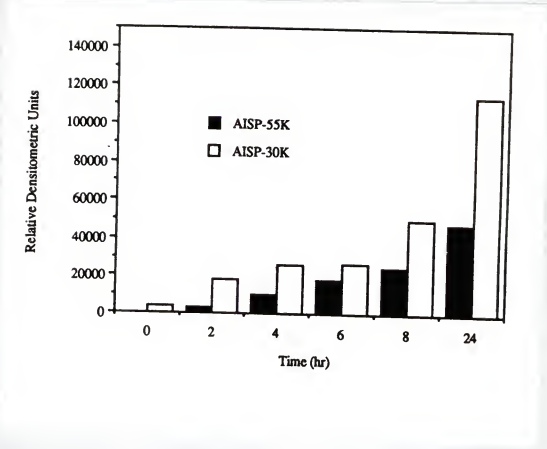

Figure 5-2. Time course of Ang II-induced secretion of AISP-30K and AISP-55K from 21-day rat brain astrocytes. As in Figure 5-1, 21-day rat brain astrocytes were treated with 1 μM Ang II for prescribed times, labeled with [^{35}S]-methionine for 8 hr and labeled secretory proteins from these cells analyzed by 2D-SDS-PAGE followed by fluorography. Relative optical densities of AISP-30K and AISP-55K are expressed and were obtained by laser densitometric scanning of fluorograms.

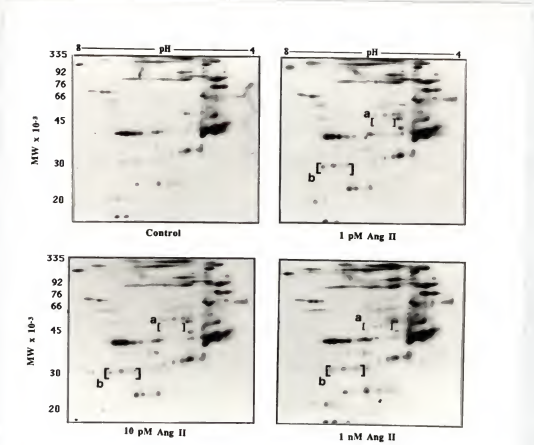

Figure 5-3A. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled proteins secreted by cultured 21-day rat brain.astrocytes in response to increasing concentrations of Ang II. Confluent 21-day rat brain astrocytes were extensively rinsed in serum-free MEM containing 10 μM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C without additions or in the presence of indicated concentrations of Ang II during the last 8 hr of the incubation period. During the final 8 hr of the 24 hr incubation period, cells were pulsed with 100μCi/dish L-[^{35}S]-methionine. Cultures were terminated, conditioned medium samples processed, and total [^{35}S]-methionine-labeled secreted proteins (100,000 cpm/gel) analyzed by 2D-SDS-PAGE followed by fluorography as described in *Methods*. AISP-55K and AISP-30K are indicated by brackets a and b, respectively.

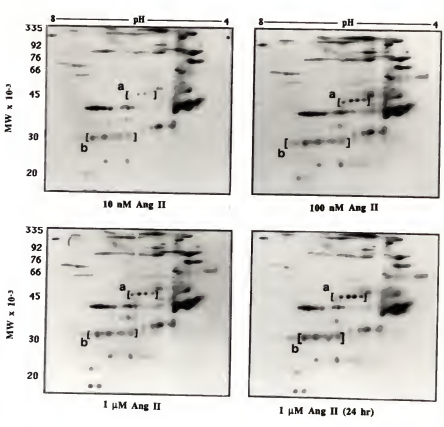

Figure 5-3B. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled proteins secreted by cultured 21-day rat brain.astrocytes in response to increasing concentrations of Ang II. Confluent 21-day rat brain astrocytes were extensively rinsed in serum-free MEM containing 10 μM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C without additions or in the presence of indicated concentrations of Ang II during the last 8 hr of the incubation period. During the final 8 hr of the 24 hr incubation period, cells were pulsed with 100μCi/dish L-[^{35}S]-methionine. Cultures were terminated, conditioned medium samples processed, and total [^{35}S]-methionine-labeled secreted proteins (100,000 cpm/gel) analysed by 2D-SDS-PAGE followed by fluorography as described in *Methods*. *Bottom, right*, Expression of AISP-30K and AISP-55K in response to treatment with 1 μM Ang II for 24 hr in this experiment is shown for comparison. AISP-55K and AISP-30K are indicated by brackets a and b, respectively.

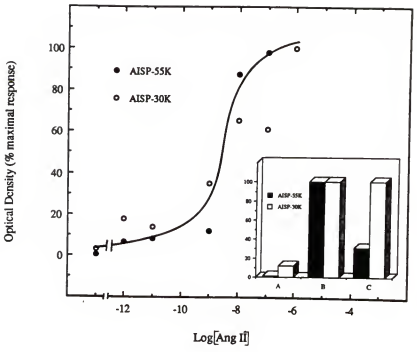

Figure 5-4. Dose response of Ang II-induced secretion of AISP-30K and AISP-55K from 21-day rat brain astrocytes. Densitometric analysis of fluorograms shown in Figure 5-3 A and B reveals that Ang II-induced synthesis and secretion of AISP-55K (•) and AISP-30K (o) is dose-dependent with an EC_{50} of 1.5 nM. *Inset*, Densitometric analysis of fluorograms similar to those shown in Fig 5-1 shows that secretion of AISP-55K (black bars) in response to 1 μM Ang II was blocked by 10 μM sar^1, ile^8 Ang II. Ang II-induced secretion of AISP-30K (white bars) was not inhibited by sar^1, ile^8 Ang II. *Column A*, untreated cultures; *column B*, 1 μM Ang II; *column C*, 1 μM Ang II + 10 μM sar^1, ile^8 Ang II

secretion of both AISP-55K and AISP-30K in 21-day rat brain astrocytes following 8 hr treatment with Ang II. Similar analysis revealed maximal stimulation of AISP-30K and AISP-55K (30 fold and 422 fold over basal, respectively) at 100 nM and higher concentrations of Ang II. Maximal stimulation observed following 24 hr treatment with 1 μM Ang II is shown in Figure 5-3B for comparison.

The presence of 10 μM sar^1, ile^8-Ang II significantly attenuated induction of AISP-55K synthesis and secretion by 1 μM Ang II (Figure 5-1 and Figure 5-4, *inset*). In contrast, synthesis and secretion of AISP-30K was not inhibited by $sar,^1$ ile^8-Ang II, and in some experiments, 10 μM and higher concentrations of this antagonist potentiated Ang II induction of AISP-30K. This effect may be due to a partial agonist activity of sar^1, ile^8-Ang II observed at these concentrations (Streeten and Anderson, 1984).

Ang II induction of AISP-30K and AISP-55K was also compared in astrocytes cultured from neonatal, 7-day, 14-day, 21-day, and adult rat brain. As shown in Figure 5-5 A, B and C, Ang II induced AISP-55K secretion was observed in astrocytes from 7-day, 14-day, 21-day, and adult rat brain. These responses, however, were not equal and appeared to progressively increase with age of the animal. Ang II-induction of AISP-55K secretion was not observed in neonatal rat brain astrocytes. Ang II-induced AISP-30K secretion was not observed in neonatal, 7-day, or 14-day rat brain astrocytes,

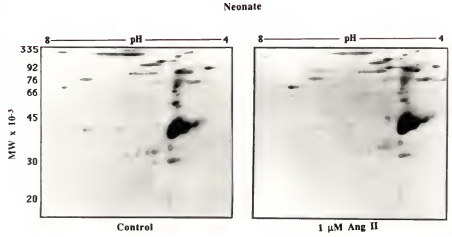

Figure 5-5A. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled proteins secreted by astrocytes cultured from neonatal rat brain in the presence of 1 μM Ang II. Confluent neonatal rat brain astrocytes were extensively rinsed in serum-free MEM containing 10 μM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C without additions (control) or in the presence of 1 μM Ang II. During the final 8 hr of this period, cells were pulsed with 100μCi/dish L-[^{35}S]-methionine. Cultures were terminated, conditioned medium samples processed, and total [^{35}S]-methionine-labeled secreted proteins (100,000 cpm/gel) analysed by 2D-SDS-PAGE followed by fluorography as described in *Methods*. AISP-55K and AISP-30K are indicated by brackets a and b, respectively.

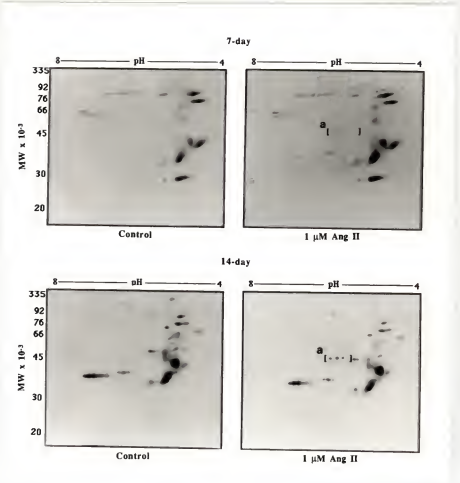

Figure 5-5B. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled proteins secreted by astrocytes cultured from 7-day and 14-day rat brain in the presence of 1 μM Ang II. Confluent 7-day and 14-day rat brain astrocytes were extensively rinsed in serum-free MEM containing 10 μM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C without additions (control) or in the presence of 1 μM Ang II. During the final 8 hr of this period, cells were pulsed with 100μCi/dish L-[^{35}S]-methionine. Cultures were terminated, conditioned medium samples processed, and total [^{35}S]-methionine-labeled secreted proteins (100,000 cpm/gel) analysed by 2D-SDS-PAGE followed by fluorography as described in *Methods*. AISP-55K and AISP-30K are indicated by brackets a and b, respectively.

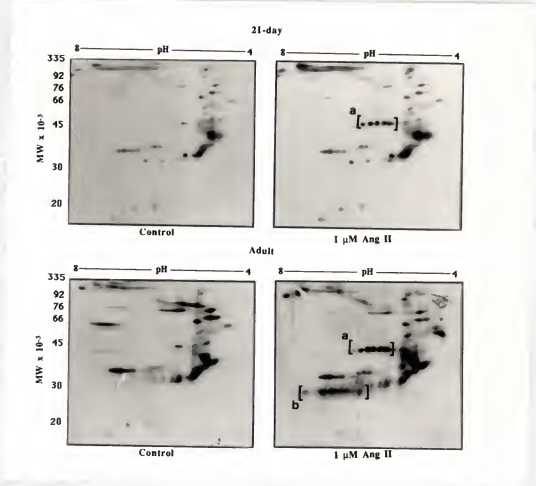

Figure 5-5C. Representative fluorograms obtained following 2D-SDS-PAGE of [^{35}S]-methionine-labeled proteins secreted by astrocytes cultured from 21-day and adult rat brain in the presence of 1 μM Ang II. Confluent 21-day and adult rat brain astrocytes were extensively rinsed in serum-free MEM containing 10 μM L-methionine (1/10th normal) and incubated in this same medium for 24 hrs at 37° C without additions (control) or in the presence of 1 μM Ang II. During the final 8 hr of this period, cells were pulsed with 100μCi/dish L-[^{35}S]-methionine. Cultures were terminated, conditioned medium samples processed, and total [^{35}S]-methionine-labeled secreted proteins (100,000 cpm/gel) analysed by 2D-SDS-PAGE followed by fluorography as described in *Methods*. AISP-55K and AISP-30K are indicated by brackets a and b, respectively.

was minimal in astrocytes from 21-day rat brain, and was robust in astrocytes from adult rats.

Sequence determination and identification of AISP-30K & 55K.

To better understand the role of AISP-55K and AISP-30K in the action of Ang II on astrocytes, N-terminal amino acid sequence identification of these proteins was attempted. Following concentration, PAGE-separation, and transfer of the Ang II-induced secretory proteins to Immobilon™ membranes, N-terminal amino acid microsequencing was performed on two separate AISP-55K protein spots (Figure 5-1, arrows 1 and 2) and a single AISP-30K protein spot.

Figure 5-6 shows sequence data for the first 21 N-terminal amino acids from AISP-55K (Fig. 5-1, arrow 1). Sequence determination of a second AISP-55K spot (Fig. 5-1, arrow 2) yielded 13 N-terminal amino acids and demonstrates identity to the other AISP-55K spot sequenced (data not shown). This analysis indicated that for AISP-55K variation in pI values between individual spots within this group most likely represent different degrees of post-translational modification of a single protein. Sequence data from AISP-55K were compared to reported NBRF and Genbank sequences using both FASTA and TFASTA database search programs and indicated that 21 amino acids of the N-terminus of AISP-55K have 100% identity to amino acids 24-44 of rat plasminogen activator inhibitor (rPAI-1), a M_r 50-55,000 glycoprotein

```
                    10                  20
AISP-55K    SPLPESHTAQ QATNFGVKVF G
            :::::::::: ::::::::::
rPAI-1      SPLPESHTAQ QATNFGVKVF Q
                    30                  40

                    10                  20
AISP-30K    CSCAPTHPQT AFCBSBLVIX A
            :.:.:.:::: :::.:.:::  :
hTIMP       CTCVPPHPQT AFCNSDLVIR A
                    30                  40
mTPA-S1     CSCAPPHPQT AFCNSDLVIR A
                    30                  40
mG-R 16C8   CSCAPPHPQT AFCNSDLVIR A
                    30                  40
```

Figure 5-6. Comparison of N-terminal amino acid sequences of AISP-55K and AISP-30K to rPAI-1 and hTIMP/mTPA-S1/hG-R 16C8, respectively. Secretory proteins from 21-day rat brain astrocyte conditioned medium were concentrated, resolved by 2D-SDS-PAGE and transfered to Immobilon™ membranes. N-terminal amino acid microsequencing of AISP-55K and AISP-30K was performed and sequences compared to database entries using the FASTA and TFASTA programs of Pearson and Lipmann . *Top*, N-terminal sequence comparison of amino acids 1-21 of AISP-55K to amino acids 24-44 of rat plasminogen activator inhibitor (rPAI-1) (Zeheb and Gelehrter, 1988) indicates 100% sequence identity (indicated by :) between these two regions. Amino acids 1-23 of rPAI-1 constitute the cleaved signal peptide. *Bottom*, comparison of N-terminal amino acids 1-21 of AISP-30K to amino acids 25-45 of human tissue inhibitor of metalloproteases (hTIMP) (Docherty *et al.*, 1985), mouse phorbol ester-induced protein S1 (mTPA-S1) (Johnson *et al.*, 1987) and mouse growth-responsive gene 16C8 (mG-R 16C8) (Edwards *et al.*, 1986) indicates 72, 81 and 81% sequence identity between AISP-30K and these proteins, respectively. AISP-30K shares 100% homology within these regions if conserved amino acid substitutions (indicated by .) are considered. Amino acids 1-24 of hTIMP, hG-R 16C8 and mTPA-S1 constitute the cleaved signal peptides of these proteins.

reported to be secreted by several cell types (Zeheb and Gelehrter, 1988; Danø *et al.*, 1985). Amino acids 1-23 of PAI-1 constitute the cleaved signal peptide of this protein.

N-terminal amino acid sequencing of one AISP-30K spot (Fig. 5-1, arrowhead) resulted in identification of 20 of the first 21 N-terminal amino acids of this protein. Comparison of this sequence to database sequences (Fig. 5-6) indicated that amino acids 1-21 of this region of AISP-30K have 72% identity to amino acids 25-45 of human tissue inhibitor of metalloproteases (hTIMP) (Docherty *et al.*, 1985) and 81% identity to amino acids 25-45 of both a rat phorbol ester-inducible protein (rTPA-S1) (Johnson *et al.*, 1987) and the murine growth-responsive protein 16C8 (mG-R 16C8) (Edwards, *et al.*, 1986). Amino acids 1-24 of these proteins constitute the cleaved signal peptide. The proteins hTIMP, mTPA-S1 and mG-R 16C8 have been reported to exhibit M_r values of 22-29,000, depending upon the degree of glycosylation, and are thought to be identical or closely related. AISP-30K shares 100% homology with these proteins at the N-terminus if conserved amino acid substitutions are considered.

Discussion

Experiments described here demonstrate that astrocytes cultured from brain of developing and mature rats secrete PAI-1 and a TIMP-related protein in response to stimulation by angiotensin II. Assignment of AISP-55K as PAI-1 is likely

correct based upon N-terminal sequence homologies and molecular weight and isoelectric point correlations between AISP-55K and those reported for PAI-1 (Andreasen *et al.*, 1986; Van Mourik *et al.*, 1984). Based upon similar criteria, AISP-30K is most likely a rat analogue of TIMP or a closely related protein (Docherty *et al.*, 1985; Johnson *et al.*, 1987; Edwards *et al.*, 1986; Mawatari *et al.*, 1989). Ang II-induced synthesis and secretion of both proteins was dose-dependent with an EC_{50} of 1.5 nM and blocked in the presence of the Ang II receptor antagonist, sar^1, ile^8-Ang II, indicating that this effect is Ang II receptor mediated. Synthesis and secretion of AISP-30K was not blocked by this antagonist, and in some cases was potentiated by sar^1, ile^8-Ang II suggesting that induction of this protein may be due to a selective partial agonist effect of sar^1, ile^8-Ang II, mediated by a functionally distinct subset of Ang II receptors. Since neonatal rat brain astrocytes did not secrete either PAI-1 or TIMP-related protein in response to Ang II and since this effect increased progressively with age of the animal from which astrocytes were cultured, it appears that responses to Ang II may be developmentally regulated in this cell type.

Several intriguing possibilities relate to the physiological significance of Ang II-induced, coordinated secretion of PAI-1 and TIMP-related protein by astrocytes of brain. Protease nexin I (Geunther *et al.*, 1985; Gloor *et al.*, 1986; Gurwitz *et al.*, 1988), a glial-derived serine protease inhibitor (*serpin*) closely related to PAI-1, has

been shown to regulate astrocyte proliferation as well as neurite outgrowth in neuroblastoma cells. It is tempting to postulate that Ang II may have similar glial regulatory or neurotrophic properties in the brain mediated by glial-derived PAI-1 and/or TIMP-related protein. This is a plausible hypothesis given observations that plaminogen activator and activator inhibitor activity have been associated with neurite outgrowth in neuroblastoma cells (Benjamin *et al.*, 1989; Soreq *et al.*, 1983) as well as correlations between plasminogen activator and inhibitor activity and tumorigenicity of cultured human glioma cells (Gross *et al.*, 1988). Alternatively, close approximation of astrocyte processes to endothelial cells of the cerebral vasculature may allow for astrocyte secretory proteins to influence these cells and/or fibrinolytic states in the brain. Astrocytes have been shown to induce blood-brain barrier tight junctions in endothelial cells (Janzer and Raff, 1987). Observations that peripheral vascular smooth muscle cells possess Ang II receptors (Aguliera and Catt, 1981) and have been shown to secrete PAI-1 in response to other peptides (McFall *et al.*, 1990) is suggestive of an analogous function in astrocytes. Finally, secretion of rPAI-1 and TIMP-related protein may mediate a growth response to Ang II in these cells. Although little is known of Ang II action on astrocytes, other agents linked to protein kinase C activation, including tetradecanoyl phorbol acetate (TPA) (Murphy *et al.*, 1987) and muscarinic cholinergic agonists

(Ashkenazi *et al.*, 1989), have been shown to be mitogenic to cultured astrocytes. Interestingly, TPA-S1, a protein closely related or identical to TIMP, is the product of a growth-responsive gene induced by a number of cell mitogens including TPA (Edwards *et al.*, 1986), epidermal growth factor (Mawatari *et al.*, 1989), and interleukin-1 (Murphy *et al.*, 1987). At present it is not clear whether these protease inhibitors have direct effects on cells of the CNS or act to alter extracellular matrix integrity through the modulation of endogenous proteases.

It is of particular interest to compare the different response of neonatal and 21-day rat brain astrocyte cultures to Ang II. Several possibilities may explain these observations. First, developmental plasticity of the mammalian CNS likely dictates age-dependent changes in cell responses to extracellular cues. These changes may include developmentally-regulated synthesis and secretion of proteins by astrocytes in response to Ang II. This is an interesting hypothesis in light of evidence demonstrating developmentally regulated muscarinc receptor responses in fetal and neonatal astrocytes (Ashkenazi *et al.*, 1989). Similarly, Ang II receptors may mediate several developmentally timed processes via differential coupling to their effector systems. This is a particularly intriguing possibility in light of recent demonstration of Ang II receptors in developing fetus (Millan *et al.*, 1989). Alternatively, it is possible that our culture technique may select for a particular subset of Ang

II-sensitive astrocytes in 21-day rat brain versus the neonate. It is unlikely that 21-day rat brain astrocyte cultures contain a significant percentage of Ang II-responsive non-astrocytic cells because: (*i*) 95-98% of cells in these cultures stain positively for the astrocyte-specific GFAP; (*ii*) 2D-SDS-PAGE analysis of cellular proteins from neonate and 21-day rat brain astrocytes demonstrate largely homologous profiles (Figure 3-2); and (*iii*) properties of astrocytes fron neonatal and adult brains have been suggested to be similar (Noton and Farooq, 1989).

Although Ang II-induced TIMP-related protein and PAI-1 synthesis and secretion has been principally characterized in cultures derived from brains of neonatal and 21-day-old rats, preliminary observations suggest that astrocytes from postnatal day 7 and older, synthesize and secrete PAI-1 in response to Ang II, whereas only astrocyte cultures derived from 21-day and adult animals demonstrate Ang II-induction of TIMP-related protein. These are interesting observations considering differential effects of sar^1, ile^8-Ang II on Ang II-induced secretion of these proteins. If induction of these two proteins are mediated by different subtypes of Ang II receptors, then age-dependent changes in expression of these subtypes may explain different developmental "windows" of protease inhibitor induction by Ang II. Furthermore, different responses of these receptor subtypes to sar^1, ile^8-Ang II may explain the partial agonist activity of this antagonist suggested by these and other studies. Recent

demonstration of Ang II receptor subtypes using nonpeptide Ang II antagonists gives credence to this hypothesis. Future availability of these nonpeptide Ang II antagonists may allow this hypothesis to be tested.

In one experiment, treatment of 21-day rat brain astrocytes with 10 μM Ang II for 6 hr in the presence of 1 μg/ml cycloheximide failed to induce secretion of labeled PAI-1, while Ang II-induced secretion of labeled TIMP-related protein into culture medium was slightly enhanced. In addition, co-incubation of 10 μM Ang II with 1 μM actinomycin D in this experiment did not alter Ang II-induced secretion of labeled TIMP-related protein or PAI-1 by 21-day rat brain astrocytes (data not shown). Although preliminary, these observations may suggest differential regulatory mechanisms controlling release of protease inhibitors from these cells.

Finally, if Ang II-induced synthesis and secretion of rPAI-1 and the TIMP-related protein by astrocytes is indeed age-dependent, then it is possible that control of extracellular proteolytic activity in the CNS may regulated by locally produced hormonal factors acting on glia during development and/or aging. Recent identification of other protease inhibitors present in brain amyloid deposits in Alzheimer's disease (Abraham *et al.*, 1988; Abraham, 1989; Wagner *et al.*, 1989) indicate that control of protease activity may indeed be important to both normal and pathological processes in the aging brain. At present,

however,the age-dependent nature of protease-protease inhibitor interactions in the brain is not understood.

CHAPTER 6
SUMMARY AND DISCUSSION

Growth, differentiation, and maintenance of neurons, oligodendrocytes and astrocytes of the central nervous system are now known to depend upon close associations between these cells in the brain. Hypotheses concerning cell-cell interactions in the brain often presumed the necessity of direct contact between participating cells, yet as exemplified by neurochemical transmission at the synaptic cleft, this need not always be the case. It is now clear that in many circumstances intercellular communication in the brain depends more upon upon biochemical intermediaries secreted by these cells than upon their close apposition and physical contact. It is now also apparent that CNS peptides, including several well-known growth regulatory factors, may serve as biochemical intermediaries in the brain, and that resident astrocytes play an integral role in both production of some of these factors, and regulation and/or mediation of their cellular actions during CNS development. Based upon these ideas, the goal of this project was to investigate both constitutive and hormone-induced synthesis and secretion of proteins by astrocytes of the postnatal developing rat brain. It was hoped that information gained from these studies would

shed light on intercellular communication within the developing and maturing mammalian CNS.

Studies presented in Chapter 3 were designed to investigate potential age-dependent changes in the constitutive secretion of proteins by astrocytes. These studies demonstrate that astrocytes cultured from the maturing rat brain differentially synthesize and secrete the insulin-like growth factor (IGF) binding protein, IGFBP-2. Specifically, astrocytes cultured from brains of rats postnatal day 14 and older synthesize and secrete high levels of this binding protein, as compared to their counterparts from neonatal (1-day) and 7-day rat brain. Northern analysis of total cellular RNA isolated from neonatal and 21-day rat brain astrocytes using a specific probe for IGFBP-2 support protein data, demonstrating that astrocytes cultured from 21-day rat brain express 6-fold higher levels of IGFBP-2 mRNA than do those from neonatal animals. Positive correlation between protein and mRNA expression in these astrocytes indicates that IGFBP-2 expression in these cells may be regulated at the level of gene transcription. Failure to detect appreciable levels of IGFBP-2 protein in neonatal and 21-day astrocyte cell lysates further indicates that this protein is not stored at high levels inside these cells. Synthesis and secretion of this binding protein also seems confined to astrocytes from developing brain since IGFBP-2 was not detected in conditioned medium from cultures of neonatal rat brain neurons by Western blotting. These

observations do not rule out the possibility that these neurons may synthesize and secrete this protein at levels too low for detection by our antiserum. In support of a neuronal source of this binding protein, very small amounts of IGFBP-2 mRNA were detected in cultured neonatal rat brain neurons following overexposure of film; however, contaminating astrocytes in these cultures may explain such observations.

These studies also demonstrate that synthesis and secretion of IGFBP-2 by 21-day rat brain astrocytes is stimulated by exposure to dibutyryl cAMP, an agent which both inhibits proliferation and promotes differentiation of these cells. DiBucAMP-stimulated secretion of IGFBP-2 by 21-day rat brain astrocytes may also be associated with diBucAMP-induced differentiation of these cells. These observations, coupled with previous work demonstrating inhibition of IGF proliferative effects on astrocytes by IGFBPs (Han *et al.*,1988; Elgin *et al.*,1987; Busby *et al.*, 1988), lead us to believe that IGFBP-2 secretion may be a mechanism for autocrine control of IGF proliferative action in differentiated astrocytes.

The presence of insulin and the insulin-like growth factors (IGF-1 and IGF-2) in the CNS suggests that these peptides play an integral role in growth and differentiation of cells in the developing brain. Indeed, this is the case. Both astrocytes and neuroblasts grown in the presence of IGF 1 demonstrate increased DNA synthesis (Shemer *et al.*,1987; Han *et al.*,1987). IGF-1 induces several parameters of

oligodendrocyte growth and development including oligodendrocyte-type II astrocyte (O-2A) progenitor cell differentiation (McMorris *et al.*, 1986, Masters *et al.*, 1990). IGF-1 induces expression of oligodendrocyte-specific enzymes important to the process of myelination including 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Debbage, 1986), and increases oligodendrocyte production of myelin basic protein. Thus, IGF-1 likely plays an integral role in the process of myelination in the CNS (Saneto *et al.*, 1988). Substantial evidence also points to a neurotrophic role of the IGFs in the brain during development (Ishii *et al.*, 1990). IGF-1 stimulates [^{3}H]-thymidine in neuroblastoma cell lines, increases tyrosine hydroxylase levels in sympathetic neuroblasts, and is essential for the survival of neurons in primary culture (Aizenman and de Vellis, 1987). IGF-1 also acts alone and in concert with nerve growth factor to stimulate both neurite number and outgrowth in cultured rat sympathetic and sensory neurons neuro-blastoma cells (Recio-Pinto *et al.*, 1986).

In addition to their postulated developmental role, IGFs may play important regulatory roles in the adult CNS. Mature CNS tissue and cerebrospinal fluid contain IGF-1 and 2 (Latteman *et al.*, 1989). IGF-1 peptide is localized within several adult brain tissues, and mRNA transcripts for this peptide have been demonstrated in brain (Lund *et al.*, 1986). Several regions of adult brain also contain high levels of IGF-2 mRNA (Brown *et al.*, 1986). This developmental pattern

of IGF-2 expression contrasts that of other tissues in which IGF-2 levels decline dramatically after birth. Persistance of IGF-2 message, coupled with observations that IGF-2 is present at much higher levels than IGF-1 in human brain and CSF (Hossenlopp *et al.*, 1986),suggest that IGF-2 may have a special regulatory role in the mature CNS (Tseng *et al.*, 1989). That IGF-2 stimulates DNA synthesis (Mattson *et al.*, 1986) and neurite outgrowth in human neuroblastoma cell lines (Recio-Pinto and Ishii, 1984a), decreases food intake (Lauterio-1987) and inhibits pentagastrin-stimulated gastric acid secretion via vagal pathways (Mulholland and Debas, 1988) supports such a proposal.

In tissue fluids such as CSF, the IGFs circulate bound to specific carrier proteins which themselves demonstrate specific developmental regulation. Three genetically distinct IGF binding proteins have been described and have been designated IGFBP-1, 2, and 3. IGFBP-1 was initially purified from amniotic fluid (Povoa *et al.*, 1984), IGFBP-2 from the BRL-3A liver cell line (Mottola *et al.*, 1986; Lyons and Smith, 1986 ; Brown *et al.*, 1989), and IGFBP-3 from adult serum (Martin and Baxter, 1986). These proteins not only transport IGFs, but may also regulate the actions of these peptides by altering their bioavailability at IGF receptors on target tissues (Han *et al.*,1988; Elgin *et al.*,1987; Busby *et al.*, 1988).

That astrocytes of maturing rat brain preferentially secrete IGFBP-2, not only supports a role for the IGFs in the

more developed nervous system, but further suggests that IGF action in the maturing mammalian brain may be developmentally regulated through the differential expression of IGF binding proteins. This hypothesis is supported by two pieces of evidence, in particular: 1) IGF binding proteins have been shown to potentiate as well as inhibit several tissue responses to the IGF^{s} (Clemmons *et al.*, 1990); and 2) IGFBP^{s} inhibit IGF-1-stimulated DNA synthesis in CNS astrocytes (Han *et al.*,1988). Preliminary data (1 experiment) from this laboratory may also support such a proposal: co-incubation of 21-day rat brain astrocytes with anti-IGFBP-2 anti-serum and IGF-1 appears to restore IGF-1-stimulated glucose uptake to these generally unresponsive cells. In comparison, astrocytes from the neonate synthesize and secrete relatively small amounts of IGFBP-2 and demonstrate significant increases in glucose uptake in response to IGF-1 alone. Finally, synthesis and secretion of peptide binding proteins by target-cells which serve to modulate responses to growth factors is not without precedence in the CNS. Astrocytes secrete a truncated form of the EGF receptor which inhibits the mitogenic action of EGF on these cells by competetively binding free EGF (Nieto-Sampedro, 1988).

Our results also indicate that neuronal cells from neonatal rat brain synthesize and secrete very little, if any, IGFBP-2. These observations are at variance with a previous report describing a neuronal source of this protein,detected using an antiserum raised against a similar

binding protein (Ocrant *et al.*,1989). However, our results are in agreement with those of another group (Yang *et al.*, 1990) which were obtained using an antiserum to authentic IGFBP-2. Differences in culture technique or antibody sensitivity or specificity between groups may explain these different findings. Our [^{125}I]-IGF-2 ligand blotting data indicating that neonatal rat brain astrocytes synthesize and secrete small amounts of IGFBP-2 and IGFBP-3 are in good agreement with previous reports (Han *et al.*, 1988).

Stimulation of astrocyte expression of IGFBP-2 by an agent which inhibits proliferation and promotes differentiation of these cells to their mature phenotype raises important questions as to potential roles of the IGFs in differentiated astrocytes of the mature brain. Perhaps IGF drive for astrocyte proliferation is unopposed in the early postnatal brain, and is later controlled by the presence of high levels of IGFBP-2 in the more developed CNS. Induction of such autocrine control of IGF proliferative activity in maturing astrocytes may thus be a part of an overall growth control mechanism in the developmental programme' of these cells. That IGFBP-2 expression increases dramatically in astrocytes cultured between postnatal days 7 and 14 supports such a model since astrocyte proliferation declines rapidly during the early postnatal period in the rat and remains low throughout adult life (Nieto-Sampedro *et al.*, 1987).

Our model of IGF:IGFBP-2 interaction in astrocytes of the brain is summarized in figure 6-1. Here, IGF-1 and 2 act on neurons, oligodendrocytes, and astrocytes to induce neurite outgrowth, myelin production and proliferation, respectively. IGFBP-2 is synthesized and secreted by astrocytes during development which may interact with IGF-1 and IGF-2 to either potentiate or inhibit actions of these peptides on target tissues. Finally, an agent which promotes astrocyte differentiation and maturation stimulates IGFBP-2 synthesis and secretion by astrocytes, perhaps modulating IGF action in the mature brain.

Experiments described in chapter 4 were performed to ascertain the presence of distinct receptors for angiotensin II (Ang II) on astrocytes cultured from neonatal and 21-day rat brain and to characterize biochemical responses of these cells to this CNS regulatory peptide. It was hoped that information from these studies would provide insight into the role of Ang II in astrocyte cell biology during the period of postnatal rat development.

Demonstration of locally synthesized renin, angiotensinogen, converting enzyme, Ang I and Ang II in recent years has left little doubt as to the existence of a distinct renin-angiotensin system (RAS) in the brain (Unger *et al.*, 1988). In the CNS, Ang II modulates several parameters of catecholaminergic neuron function, including neurotransmitter synthesis, re-uptake and degradation (Sumners *et al.*, 1990). It is believed that these Ang II-induced changes in

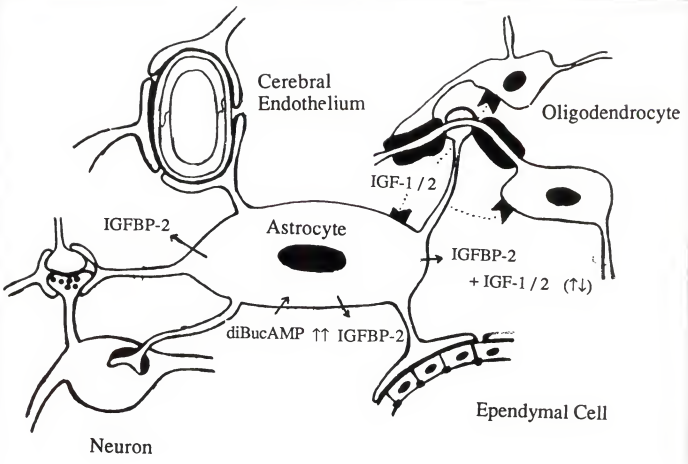

Figure 6-1. Proposed model of IGF:IGFBP-2 interactions in the developing CNS. a) IGF-1 and IGF-2 act via receptors on neurons, oligodendrocytes, and astrocytes of the brain to induce several cell-specific responses described in text. b) IGFBP-2, synthesized and secreted by astrocytes, may interact with IGF-1 and/or 2 to modulate their activity. c) Dibutyryl cAMP, a differentiaition factor for astrocytes, induces IGFBP-2 secretion in these cells, which may then directly modulate IGF action on the brain.

catecholaminergic neuron activity in relevent cardio-regulatory centers of the brain underly central dipsogenic and pressor responses to this peptide (Unger *et al.*, 1988). Thus, brain RAS is thought to play an integral role in the central control of blood pressure, and in the pathogenesis of essential hypertension (Phillips, 1983).

Recently, the existence of a RAS in astrocytes of the brain, clearly distinct from that of CNS neurons, has been demonstrated (Raizada *et al.*, 1987). As in whole brain, astrocytes from neonatal rat brain possess all components of a RAS, including Ang II receptors. Despite adequate demonstration of an astrocyte RAS, the role of Ang II in this population of cells in the postnatal developing brain has escaped definition.

This study demonstrated distinct binding sites for [^{125}I]-Ang II in astrocytes cultured from both neonatal and 21-day rat brain. Ang II receptors on astrocytes cultured from neonatal and 21-day rat brain were indistinguishable with respect to dissociation constants (0.5 nM each) and are similar in number (25 and 80 fmol/mg protein for neonatal and 21-day rat astrocytes, respectively). Competition analysis of [^{125}I]-Ang II binding to 21-day rat brain astrocytes revealed a potency profile (Ang II > sar^{1}, ile^{8}-Ang II >> Ang III > Ang I) identical to that reported for neonatal rat brain astrocytes (Raizada *et al.*, 1987). K_d and B_{max} values determined for Ang II receptors on these astrocytes are in general agreement with those described previously for both

astrocytes in culture (Raizada *et al.*,1987) and membrane-rich fractions of whole rat brain (Bennett *et al.*,1976). Results from this study also suggest that the number and affinity of Ang II receptors on astrocytes change little if at all during this early period of postnatal brain development. These findings are similar in principle, but differ somewhat in magnitude to previous studies addressing developmental changes in Ang II receptors during this postnatal period (Baxter *et al.*, 1980, Myers *et al.*, 1989). These investigators found up to ten times as many Ang II receptors on membrane and synaptosomal preparations of 28 day rat brain as compared to < 1-day-old animals.

Although Ang II receptor populations on cultured neonatal and 21-day rat brain astrocytes were pharmacologically very similar, receptor-mediated biochemical responses of these cells to Ang II were widely different. In response to Ang II, astrocytes from 21-day rat brain demonstrated significant (up to 400% over basal), dose-dependent (EC_{50} = 0.5 nM) increases in DNA synthesis, and increases in both cellular (165% over basal) and secretory (60% over basal) protein synthesis. Furthermore, Ang II-induced increases in both DNA and protein synthesis were blocked in the presence of the Ang II receptor antagonist sar^1, ile^8-Ang II, indicating that these effects are indeed Ang II receptor mediated. In contrast, astrocytes from neonatal rat brain demonstrated only a modest increase in DNA synthesis and no increase in protein synthesis in response to

Ang II even at doses 100-fold higher than those which induced these effects in 21-day rat brain astrocytes.

Results from this study thus suggest that Ang II may demonstrate growth regulatory properties in astrocytes from maturing rat brain. These observations are consistent with recent evidence indicating that Ang II may act to promote cellular growth and differentiation in peripheral tissues. Ang II increases DNA synthesis, protein synthesis and cell size, and induces expression of the growth associated proto-oncogene *c*-fos in cultured vascular smooth muscle cells (Campbell-Boswelland Robertson, 1981; Geisterfer *et al.*, 1988; Berk *et al.*, 1989; Naftilan *et al.*, 1989). Preliminary evidence from this study demonstrates similar Ang II induction of c-fos in astrocytes from 21-day rat brain. Thus, observations presented here support a growth promoting role of Ang II in astrocytes of the brain. Conclusive definition of Ang II as an astrocyte growth factor must, however, await demonstration of Ang II-induced increases in cell number. In two experiments, such an effect was not observed, but a more careful analysis including [^{3}H]-thymidine autoradiography is currently underway.

In chapter 5, astrocyte cellular responses to Ang II were further investigated in cultures of these cells from the postnatal developing brain. Experiments described address the hypothesis that Ang II effects on the brain may include induction of specific astrocyte secretory proteins which may themselves demonstrate regulatory activity in the brain. In

particular, effects of Ang II on *de novo* synthesis and secretion of proteins by neonatal and 21-day rat brain astrocytes were determined using 2D-SDS-PAGE analysis of radiolabeled proteins in growth medium conditioned by these cells . Results from these studies suggest that Ang II induces synthesis and secretion of two protease inhibitors, plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloproteases (TIMP), or a closely related protein, by astrocytes cultured from 7-(PAI-1), 14-(PAI-1), 21-day (both), and adult (both), but not neonatal rat brain. Induction of these proteins is dose-dependent (EC_{50} of 1.5 nM) and is inhibited by co-incubation with the Ang II receptor antagonist sar^1, ile^8-Ang II, again indicating Ang II receptor mediation of this effect.

PAI-1 is a member of an important class of protease inhibitory peptides which inhibit serine proteases and are collectively known as *serpins* . These inhibitors are widely distributed in tissues and are involved in several processes including fibrinolysis, blood coagulation and complement activation. Members of the serpin family include protease nexins, α_1-antichymotrypsin, C1-inhibitor and α_2-antiplasmin.

PAI-1 is one of two inhibitors (the other designated PAI-2) of the highly specific serine proteases, urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). Balance between plasminogen activators and activator inhibitors ultimately regulates levels of activated plasmin, itself a broad spectrum serine protease. Activated

plasmin, formed by u-PA or t-PA cleavage of the inactive proenzyme plasminogen, proteolytically degrades fibrin and a variety of extracellular matrix and basement membrane proteins, and may be involved in biological processes reliant upon breakdown of extracellular matrix and basement membranes, such as cell migration, invasion, tissue remodeling, and tumor metastasis. Thus, balance between u-PA, t-PA and their inhibitors PAI-1 and PAI-2, may be critical to normal cellular and tissue growth and development, whereas their imbalance may contribute to the pathogenesis of certain tissue invasive diseases

In brain, serine protease inhibitor activity has been associated with several cellular processes including promotion of neurite outgrowth, regulation of glial cell proliferation, and morphological differentiation of cells of neuronal origin. Glial-derived protease nexin-1 (GdPN-1), a *serpin* closely related to PAI-1, stimulates astrocyte proliferation and promotes neurite outgrowth from neuroblastoma cells. PAI-1 demonstrates similar activity: u-PA and PAI-1 activity are associated with neurite outgrowth in neuroblastoma cells (Benjamin *et al.*, 1989; Soreq *et al.*, 1983); and u-PA and PAI-1 activity correlate well with several tumorigenic parameters in human gliomas (Gross *et al.*, 1988). Data presented here, taken in context with the growth promoting activity of Ang II demonstrated in Chapter 4, strongly suggest that proliferative responses of 21-day

rat brain astrocytes to Ang II may involve induction of PAI-1 by this hormone.

Recent data also indiate that aberant *serpin* activity in the aging brain may underly pathological processes associated with Alzheimer's disease (Abraham *et al.*, 1988, Van Nostrand *et al.*, 1989). Deposition of "amyloid" proteins to form the core of characteristic neuritic plaques may well represent an early event in the neuropathology of Alzheimer's disease. However, these neuritic plaques are also present in much fewer numbers and in restricted distributions in the brains of most older humans (Abraham *et al.*, 1988). Thus, it appears that limited amounts of amyloid are deposited during normal aging, and is accelerated in Alzheimer's disease. Interestingly, the *serpin* a_1-antichymotrypsin is detected at high levels in brain amyloid deposits of Alzheimer's disease. This inhibitor is proposed to indirectly contribute to the formation of amyloid deposits via inhibition of endogenous serine proteases (Abraham *et al.*, 1988). Identification of another *serpin*, protease nexin II as the amyloid precursor protein found in these deposits supports the hypothesis that *serpin*-altered proteolysis contributes to the pathogenesis of Alzheimer's disease (Oltersdorf *et al.*, 1989; Van Nostrand *et al.*, 1989). Accordingly, demonstration of Ang II-induced release of PAI-1 from astrocytes of mature, but not neonatal rat brain raises exciting possibilities for the role of brain RAS in this disease.

Observations from this study further indicate that Ang II induces the release of TIMP, or a closely related protein, by astrocytes of maturing rat brain. TIMP is a specific irreversible inhibitor of metallo-proteinases, most notably interstitial collagenase and elastase, which tonically suppresses activity of these proteolytic enzymes in extracellular spaces under most normal physiological conditions (Docherty *et al.*, 1985). Evidence indicates that enhanced metalloprotease activity is important to remodeling of the extracellular matrix during normal development, growth, and tissue repair (Apodaca *et al.*, 1990), while such activity may be involved in uncontrolled degradation of the extracellular matrix in several pathological conditions, including tumor invasion and metastasis. Thus, under normal circumstances, TIMP likely plays a key role in preserving the integrity of connective tissue components of the extracellular matrix, with tissue destruction occurring following perturbations of its controlling excess.

Mechanisms governing balance between metalloproteases and their inhibitors are largely unknown. Metalloproteinase activity can be modulated by several stimuli including hormones, phorbol esters and cell morphogens (Murphy *et al.*, 1985; Apodaca *et al.*, 1990), or may be regulated by changes in levels of inhibitors such as TIMP. TIMP expression is induced by epidermal growth factor and tumor necrosis factor, and has been linked to proliferative responses in microvascular endothelial cells (Mawatari *et al.*, 1989). In

brain, TIMP has been demonstrated in fetal astrocytes and glioma cells, and is proposed to modulate growth and migration of these cell types during normal brain development and local tumor extension, respectively. Interstingly, TIMP gene sequence is identical to that of two growth associated genes, phorbol ester-inducible gene, TPA-S1, and murine growth-responsive gene, mG-R 16C8. Increased levels of mRNA transcripts for TIMP, TPA-S1, and mG-R 16C8 are detected several hours after stimulation of cells by a number of mitogens and are considered "late" elements of the growth response. Thus, TIMP secretion may be involved in either "late" events important for cell growth or may be a component of a regulatory feedback response to initial mitogenic signals. Correlation between Ang II-induced TIMP secretion and Ang II-stimulated DNA and protein synthesis described in Chapter 4 supports an association between TIMP expression and cellular growth responses. These data do not, however, imply causality between TIMP secretion and growth responses of astrocytes to Ang II, and it is not certain whether TIMP actually promotes or modulates growth in these cells. That astrocytes cultured from mature but not neonatal brain demonstrate hormonal induction of TIMP, coupled with the possibility that TIMP secretion is actually a negative feedback response to astrocyte growth, may provide clues to why astrocytes do not normally divide in adult brain

A final possibility for the role of PAI-1 and/or TIMP in the brain may actually involve regulation of brain RAS. Both

PAI-1 and TIMP are not absolutely specific for their target proteases: PAI-1 is specific for u-PA and t-PA only in the sense that it reacts slowly with other serine proteases (Andreasen *et al.*1990); and TIMP inhibits several different metalloproteases including elastase and several collagenases (Docherty *et al.*, 1985). Broad spectrum protease inhibitory activities of these proteins thus raise the possibility that TIMP and/or PAI-1 could inactivate renin, converting enzyme, or other proteins involved in regulation of these components of the brain RAS. If so, Ang II induction of these protease inhibitors could indeed be a mechanism of feedback inhibition of brain RAS by Ang II. At present this possibility remains speculative since experimental evidence in support of this hypothesis has not been described.

Developmental profiles of Ang II responsiveness in these cultured astrocytes is particularly intriguing. Results presented in Chapter 4 suggest that Ang II acts as a growth factor for astrocytes from 21-day, but not neonatal rat brain. Ang II induces PAI-1 synthesis and secretion in astrocytes cultured from rats as young as 7 days, while induction of TIMP occurs in astrocytes cultured from 21-day and adult rats. Differential responses in these cultured cells may be due to a number of factors: 1) Ang II receptors on astrocytes may be coupled to different cellular responses during development; 2) astrocytes from brain may possess different subtypes of receptors during development which are coupled to different responses; or 3) culture

techniques employed in our laboratory may select for a population of Ang II-responsive astrocytes found in brains of older, but not younger animals. Future advances in identification of subtypes of astrocytes in the CNS may distinguish between these possibilities.

A summary of data from Chapters 4 and 5 is illustrated in Figure 6-2. Ang II acts via specific cell surface receptors on astrocytes from 21-day rat brain to stimulate DNA and cellular protein synthesis. Ang II similarly causes increased protein secretion by these cells and induces the synthesis and secretion of PAI-1 and a protein either identical, or closely related to TIMP. Ang II-induced protease inhibitors may then act directly or indirectly upon neurons to stimulate neurite outgrowth, or upon astrocytes to promote or regulate proliferation. Absence of these responses in astrocytes from neonatal rat brain, despite the presence of similar Ang II receptors on these cells, suggests developmental regulation of Ang II effector systems in these cells of the brain.

In conclusion, studies presented here emphasize that astrocytes of the mammalian CNS synthesize and secrete proteins which may influence the growth, maintenance, and function of both themselves and other cells of the brain. The astrocyte repertoir of secreted proteins is not static, but appears to change during postnatal rat CNS development, perhaps imparting differences in astrocyte responses to environmental stimuli, including growth factors, during the

postnatal period. These studies also suggest that Ang II action in the mature brain may be mediated by the synthesis and secretion of specific protease inhibitors by astrocytes, and provide initial evidence that Ang II is a growth factor for astrocytes of the postnatal developing brain.

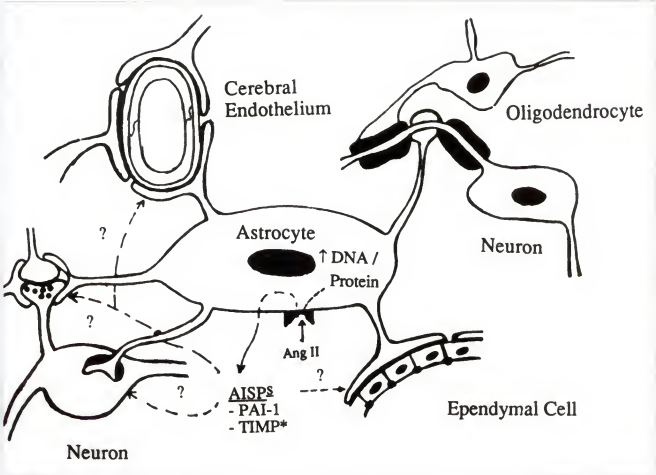

Figure 6-2. Proposed model of Ang II action on astrocytes from the developing CNS. Ang II stimulates specific cell-surface receptors on astrocytes from 21-day rat brain to stimulate DNA and protein synthesis, as well as secretion of PAI-1 and a TIMP-related protein. These protease inhibitors may then interact with astrocytes or other cells of the brain to mediate or modulate brain responses to Ang II.

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BIOGRAPHICAL SKETCH

John Ackerman Olson, Jr. was born, the eldest of three sons, to John Ackerman and Patricia Ann (Skupin) Olson on October 24, 1964. Following a relatively atraumatic childhood with his brothers Stephen and J.P. (James Patrick), John graduated from Riverview High School in Sarasota, FL in 1982. John then entered the College of Literature, Sciences and the Arts at the University of Michigan in Ann Arbor, where he earned a Bachelor of Sciences degree in cellular and molecular biology in 1986, graduating with distinction and high honors. John was subsequently accepted to the combined M.D.-Ph.D.degree program of the University of Florida. After completing two years of medical school, John began his scientific training under the guidance of Dr. Kathleen T. Shiverick of the Department of Pharmacology and Therapeutics and Dr. Mohan K. Raizada of the Department of Physiology. The degree Doctor of Philosophy will be bestowed upon John on December 22, 1990, for studies addressing the role of astrocyte secretory proteins in brain physiology. John will continue his medical-scientific training as a third-year medical student in August, 1990 and will complete the dual degree program in May, 1992. Most importantly, John will join Kimberly Ann Hillner in marriage October 26, 1991.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kathleen T. Shiverick, Chairman
Professor of Pharmacology
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1990

Dean, College of Medicine

Dean, Graduate School